THE MOLECULAR BIOLOGY OF INSECT DISEASE VECTORS

THE MOLECULAR BIOLOGY OF INSECT DISEASE VECTORS

A METHODS MANUAL

Edited by

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Foreword

Only one generation ago, entomology was a proudly isolated discipline. In Comstock Hall, the building of the Department of Entomology at Cornell University where I was first introduced to experimental science in the laboratory of Tom Eisner, those of us interested in the chemistry of life felt like interlopers. In the 35 years that have elapsed since then, all of biology has changed, and entomology with it.

Arrogant molecular biologists and resentful classical biologists might think that what has happened is a hostile take-over of biology by molecular biology. But they are wrong. More and more we now understand that the events were happier and much more exciting, amounting to a new synthesis. Molecular Biology, which was initially focused on the simplest of organisms, bacteria and viruses, broke out of its confines after the initial fundamental questions were answered – the structure of DNA, the genetic code, the nature of regulatory genes – and, importantly, as its methods became more and more generally applicable. The recombinant DNA revolution of the 1970s, the development of techniques for sequencing macromolecules, the polymerase chain reaction, new molecular methods of genetic analysis, all brought molecular biology face to face with the infinite complexity and the exuberant diversity of life. Molecular biology itself stopped being an isolated discipline, preoccupied with the universal laws of life, and became an approach to addressing fascinating specific problems from every field of biology. One example has been the birth of the discipline of molecular entomology, and with it the subdiscipline of molecular insect vector biology.

Insects are the most successful and most diverse group of higher animals, in all environments except the sea. Important features of their success are their short life cycle, their ability to evolve rapidly to exploit new niches, and their capacity to separate the growth and dispersal phases of their life cycle. Thus, they are adept at exploiting the huge food resources of agriculture – concentrated cultivation of plants and animal husbandry by humans – as well as the ready-made source of protein represented by animal or human blood. Agricultural insect pests are ubiquitous and costly exploiters of our efforts to feed and clothe ourselves. Haematophagous insects are serious threats to our health, and that of our animals, by virtue of the viruses and parasites (protozoa or worms) that they happen to carry.

To fight insect pests we must know their secrets. For more than a century Entomology has represented the effort of humans to find weak points and disarm our small, but formidable, enemies. We have learned a great deal about them, and it has been very useful. To name only one example, yellow fever was controlled by understanding the habits of the *Aedes aegypti* mosquito just as much as by the development of a vaccine against the virus. Much remains to be done, however. Parasitic diseases, and especially malaria, remain among the most important health hazards in much of the world. New or modified agricultural pests continue to erupt, as easy transport brings new insect species in contact with new food sources, and as traditional insect pests stay one step ahead of us by rapidly evolving resistance to pesti-

cides. When coupled with the knowledge accumulated by classical entomology, the new tools of molecular biology promise much help for our side. Again one example: understanding the population biology of malaria mosquitoes is a prerequisite for their control, and molecular genetic markers are now indispensable tools for the study of populations.

But the new synthesis works both ways: molecular biology helps entomology, but also *vice versa*. The best model species for studying the biology of complex organisms is the fruitfly, *Drosophila melanogaster*. And the enormous amount of accumulated information about *Drosophila* makes insects especially attractive for those who want to study fundamental biology using the comparative approach. Again one example: in the last 15 years we have understood in outline how genes control the embryonic development of *Drosophila*, and have discovered to our amazement that many of these same genes are also important for very different animals, including ourselves. More and more molecular developmental biologists are studying the regulatory genes in other insects, to understand the subtleties of how they operate. Why not do it in insects that are pests, thus contributing at the same time to learning about our enemies?

Molecular entomology is a rapidly growing and exciting field, whether we are in it for basic biological interests or for reasons of applied biology. The editors of this book have made a wonderful contribution to the progress of this field, by approaching it from the synthetic viewpoint. They have addressed the complementary needs both of those entering the field from entomology and those coming from molecular biology. In so doing, they have put together a unique book of methods, each presented at the level of practical detail needed by new practitioners. The first two sections introduce us to the culture of insect vectors of disease, and their experimental infection with parasites. The next three sections present basic methods of molecular biology, genomic mapping and molecular identification techniques as applied to insects of human importance. The final three sections deal with the key special topics of DNA transfer to insects (*via* viruses or direct addition), culture of the most important insect organs and specialized cell types, and the study of insect symbionts. The overall logic and the extent and the quality of coverage of the book are admirable. Whether you are new trainees or researchers in this field, enjoy!

Fotis C. Kafatos
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Preface

This manual aims to provide a source book of detailed protocols covering the important techniques used in molecular entomology. Although the research literature contains descriptions of practical procedures, extensive practical detail is usually not presented; more particularly, an idea of the problems that can be encountered and how they are identified and overcome is rarely included. Each chapter of this volume concentrates on one specific method, written in such a way that a competent scientist who is unfamiliar with the method can carry out the technique successfully, either at the first attempt, or with a little practice, by simply following the detailed procedures. The intention was that the methods book would form part of WHO/TDRs strategic research initiative in this area and provide a valuable resource to assist in its development. We are particularly grateful to the various experts in these specialized fields who agreed to contribute to the manual. We also greatly appreciate the fact that some of our collaborators 'volunteered' to contribute chapters describing general methods!

Our target audience includes entomologists with no experience of molecular biology, and molecular biologists interested in entering the field (who have little experience of applying the technology to insect vectors). We also hope that it will prove useful to those organizing or participating in training courses in the molecular biology of disease vectors or molecular entomology in general. Finally, researchers in related fields, such as agricultural entomologists, insect physiologists, etc., may also find some of the methods and techniques applicable, or of interest, to their own field of research.

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Abbreviations

A	Amps
A_{260}	absorbance at a wavelength of 260 nanometre
ACD	acid/citrate/dextrose
APS	ammonium persulfate
ATP	adenosine triphosphate
bp	base pair(s)
BSA	bovine serum albumin
cDNA	complementary DNA
CHX	cycloheximide
cm	centimetre(s)
cpm	counts per minute
Da	Dalton(s)
ddH_2O	double-distilled deionized water (or equivalent)
ddNTP	2′,3′-dideoxynucleotide triphosphate(s)
DEPC	diethylpyrocarbonate
diam	diameter
DMSO	dimethyl sulfoxide
DNase	deoxyribonuclease
dNTP	deoxynucleotide triphosphate(s)
20E	20-hydroxyecdysone
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunoassay
EtBr	ethidium bromide
EtOH	ethanol
FCS	fetal calf serum
g	gram(s)
g	gravitational force
G	gauge
h	hour(s)
HAP	hydroxyapatite
HBSS	Hank's balanced salt solution
HEPES	N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid
IPTG	isopropyl-β-D-thiogalactopyranoside
IU	International unit(s)
kb	kilobase pair(s)
kDa	kilo Dalton(s)
L	litre(s)
L:D	light:dark photoperiod
L3	third-stage larva(e)
m	metre(s)
M	molar

mCi	milliCurie(s)
MEM	modified Eagle's medium
mff	microfilaria(e)
mg	milligram(s)
min	minute(s)
mL	millilitre(s)
mm	millimetre(s)
mM	millimolar
mmol	millimole(s)
moi	multiplicity of infection
mRNA	messenger RNA
NBT	nitroblue tetrazolium
NCS	new born calf serum
ng	nanogram(s)
nL	nanolitre(s)
nm	nanometre(s)
nM	nanomolar
nmol	nanomole(s)
PB	phosphate buffer
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pfu	plaque forming units
pg	picogram(s)
PGI	positive gland index
pM	picomolar
polyA	polyadenylic acid
RACE	rapid amplification of cDNA ends
RAPD	random amplified polymorphic DNA
RBC	red blood cells
RFLP	restriction fragment length polymorphism
RH	relative humidity
RLO	rickettsia-like organism(s)
RNase	ribonuclease
rpm	revolutions per minute
rRNA	ribosomal RNA
RT	room temperature
RT-PCR	reverse transcription PCR
S	Svedburg unit(s)
s	second(s)
SDS	sodium dodecyl sulphate
SSC	saline sodium citrate
SSCP	saline sodium citrate phosphate
TBE	Tris/borate/EDTA buffer
TI	transmission index
Tris	2-amino-2(hydroxymethyl)-propane-1,3-diol
tRNA	transfer RNA
μCi	microCurie(s)
μg	microgram(s)
μL	microlitre(s)

μm	micrometre(s)
μM	micromolar
μMole	micromole(s)
U	unit(s)
UV	ultra-violet
v/v	volume per volume
V	Volts
Vg	vitellogenin
vol	volume
VSG	variant surface glycoprotein
w/v	weight per volume
W	Watts
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
x*g*	times gravitational force

Part One
Care and Maintenance of Insect Colonies

CHAPTER ONE

Care and maintenance of anopheline mosquito colonies

Mark Q. Benedict

1.1 INTRODUCTION

Successful maintenance of a large colony of 'wild' anophelines, or numerous small-scale genetically unique strains, depends largely on the meticulous attention of skilled individuals. However, the methods we describe below have proven to be robust, greatly reducing the level of experience and judgement required for success. Although developed with *Anopheles gambiae* in mind, we, and others, have used essentially the same methods successfully with *An. arabiensis, An. albimanus, An. stephensi, An. freeborni* and *An. quadrimaculatus.*

Numerous other methods for rearing anopheline species have been compiled (Gerberg *et al.*, 1994) and an excellent overview is recommended (Foster, 1980). For general insect rearing information and considerations, including maintaining genetic variation, the reader is referred to King and Leppla (1984) and Singh and Moore (1985).

No attempt will be made to describe methods for establishing laboratory colonies from wild-caught anophelines. This is a very labour-intensive endeavour, generally requiring intimate knowledge of the natural history of the species being colonized, and access to large numbers of field-collected mosquitoes. The reader is referred to the specialized literature on this topic (e.g. Chapman and Barr, 1969; Gahan, 1966) and those who have successfully colonized the anopheline of interest.

1.1.1 General rearing conditions

Anophelines can be reared in temperature, light, and humidity controlled incubators, or insectary rooms set aside for this purpose. Good air conditions are 27–28°C and approximately 80% humidity (Note 1). Air temperatures in this range will result in larval water temperatures approximately 1–2°C lower in covered containers. A 12 h:12 h light:dark cycle is desirable with gradual sunset and sunrise light transitions, although *An. gambiae* colonies seem tolerant of reasonable light perturbations.

Larval and adult container designs vary widely. The choice will be determined by availability and preference, although several factors should be considered. A large

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surface area is desirable for larval rearing with a water depth of no more than 2 cm. Popular containers are porcelain-coated metal trays, plastic trays, and plastic shoe boxes (Figure 1.1). Furthermore, a cover should be provided, the container must be easily cleaned, and preferably be sterilizable (Note 2). Likewise, the choice of adult cages will depend largely on preference and availability (Figure 1.2). Adult mosquitoes drip 'urine' and some species also drip blood while feeding. Unless absorbed, these fluids can create stickiness in which adults may die and fungi grow. It is desirable to provide an absorbent surface on the bottom of the cage to reduce this problem. Also, extremely small containers will limit the number of adults one can contain, and in small containers (<0.5 L) mating may be inhibited.

1.1.2 Optimizing larval culture

Optimal larval culture conditions can be identified by two easily recognized factors: (i) larval size and stage uniformity (evident especially as synchronous pupation) and, (ii) adult longevity. Under ideal conditions, all larvae will pupate synchronously over one or two days. Although this is only occasionally achieved, if >95% of pupae have not formed within a 4-day period, suboptimal conditions are indicated and culture modifications should be made. Furthermore, optimally reared adults will live much longer than poorly reared ones. Under favourable conditions, it is common for some grandparents to be alive when adult grand-progeny eclose.

A common error in larval culture is overcrowding. This results in an irreversibly wide distribution of rate of development. Even a single day of crowding has noticeable effects on development, so daily attention to the larval conditions is important to avoid this problem.

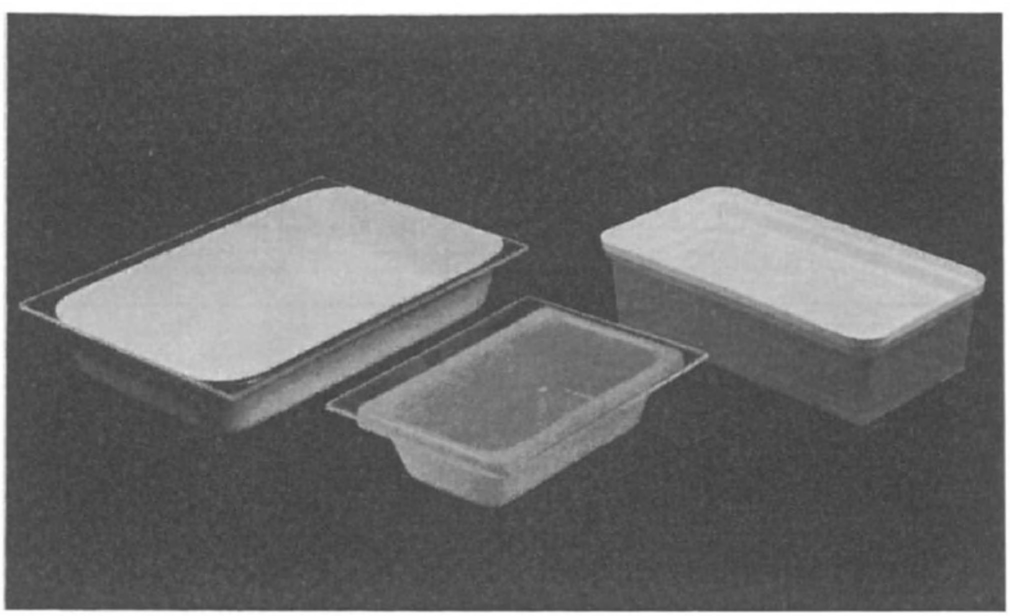


Figure 1.1 Typical larval rearing containers. Left to right are shown a porcelain-coated metal pan, a small autoclavable plastic tray, and a shoe-box type plastic container supplied with a lid. The first two are shown covered with plastic sheets of Plexiglass. The size of the container is generally matched to the stage and number of larvae present. Approximate numbers of fourth-instar larvae recommended for containers from left to right are: 150–400, 10–50, 50–150.

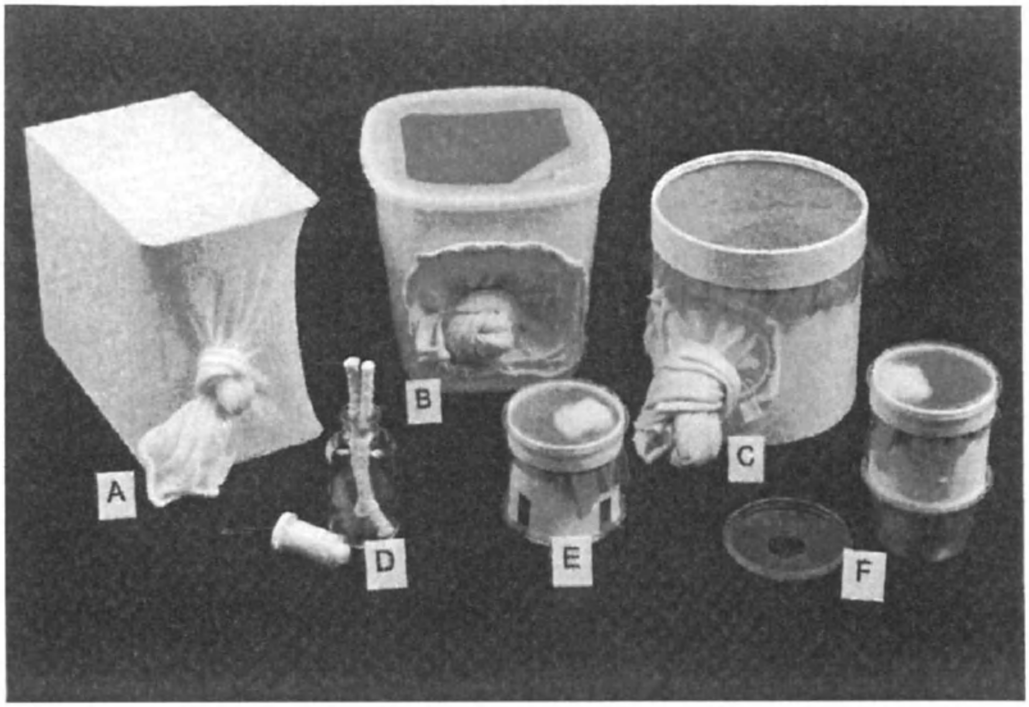


Figure 1.2 Examples of several types of adult cages and sugar sources. Adult containers can be constructed of any material that can be cleaned or discarded when soiled, has a sealable opening for transferring adults, egg dishes, and pupae, and a screen through which adults can feed on blood and sugar. (A) Tubegauze stretched over an aluminum frame. (B) A plastic bucket with a hole sealed by Tubegauze stapled in the opening and a screened removable lid. Pupae and egg dishes can be introduced through the side opening. (C) A large paper can with a screen-sealed top through which adults can feed on blood and sugar. Cages A–C will hold approximately 400 adults. (D) Examples of standing and hanging sugar-water sources; a cotton-filled plastic vial with a hole cut in the bottom, and a glass bottle with cotton wicks. (E) A small paper cage for adults with the bottom removed and replaced with a clear lid lined with filter paper. This cage will hold up to 100 adults. A sugar-water soaked pad is underneath the top lid. The screen has a small slit under the pad through which adults can be aspirated. (F) A small cage as in E set up for adult emergence or egg-laying. Emerging adults will fly up through the hole in the transparent lid and rest in the upper cup. Gravid females will fly down through the same type of lid to oviposit. The type of cup beneath, when lined with filter paper, serves as an egg-laying dish for all cages shown (Gerberg *et al.*, 1994 gives numerous other cage designs).

The most delicate and troublesome stages are invariably early instars. Especially during these stages, uncontrolled microbial growth often results in foul water, scum formation, and consequent larval death. Unfortunately, addition of sufficient food for optimal larval growth is conducive to these problems. Therefore, one must depend on larval consumption of microbes and other food to limit microbe abundance. On the other hand, third and fourth-instar larvae are able to limit microbial growth quite effectively when exposed to higher food concentrations since they filter and forage a larger volume of water. At all stages, a balance must be reached wherein the food is limited to that which larvae can consume daily, but be abundant enough that they are not stunted by insufficient food.

There are two strategies that are consistently useful to keep the larval/food density appropriate. Each recognizes the increasing suspended matter-clearing ability of more mature larvae. The first is to use one size of container for all stages of development, but to begin the cultures at a high density, and to reduce the number of larvae

in the container every couple of days by subdivision or discarding excess larvae. The high density of early stage larvae enables them to clean the water effectively, but by thinning, later stages will not be crowded detrimentally. A second approach is to start the larval culture in a small container and to use larger containers as development proceeds. This strategy is especially useful for genetic crosses and isofemale lines in which every individual is valuable.

Rearing water can come from any reliable source. We have not observed detrimental effects of municipal chlorinated or fluoridated water on anophelines. Good quality untreated groundwater, distilled, and deionized water are also usable. Preliminary comparisons of different water sources should be performed, but whatever source is chosen, assurance of consistent quality is a requisite.

1.1.3 Genetic considerations

Since a primary value of mosquito stocks is their genetic composition, stocks that are contaminated are of little worth. Stock purity depends primarily on conscientious, meticulous attention to detail. This can be accomplished only by methodology if strains are not phenotypically distinct. In this case, it may be impossible to determine that they have become contaminated so, if possible, work with phenotypically marked stocks. For this purpose, recessive alleles are preferable since contamination can quickly be observed and may be easily remedied. It may be acceptable to quickly purify polymorphic 'wild' strains for some character such as the common *stripe* (Fritz *et al.*, 1989) or *collarless* alleles (Mason, 1967). However, even using the most careful methods, contamination will sometimes occur. Therefore, if it is not possible to work with phenotypically distinct strains, the best prevention is physical isolation of each stock in a separate room. Since many laboratories maintain more than one species of *Anopheles*, special care should also be taken to prevent cross-species contamination (Note 3).

Two different culture methods are presented below in the form of rearing schedules beginning with blood-feeding since this event determines the timing of all subsequent activities. The first method is for relatively large-scale culture of stocks (>100 adults) that will not be reared as isofemale lines. This method is suggested for low-fertility strains, or in instances where high polymorphism or large numbers are needed. The second is oriented toward genetic applications on a smaller scale with isofemale rearing. This method is recommended for routine culture of a large number of genetically unique fully fertile strains. However, components and methods of the two can be combined, such as mass-egging small cultures, and isofemale lines derived from large-scale colonies. Most of the materials are common to both methods, and each results in a 2.5 week generation time.

1.2 MATERIALS

1. Larval food: A 2% (w/v) slurry of 2 parts TetraMin Baby-E fish food (TetraWerke, Germany): 1 part brewer's yeast (w/w) in water (Note 4). Make only enough slurry for 3 days and store at 4°C. Stir slurry vigorously between each pipetting. The 2:1 powder should be premixed for convenience and frozen along with any unmixed TetraMin Baby-E fish food.
2. Paper cups (e.g. Fonda (St. Albans, Vermont, USA) # 00001-30106, 1 U.S. pt.; Note 5; Figure 1.2).

3. Sweetheart™ (Chicago, Illinois, USA) ME8RX clear plastic cups.
4. Sweetheart™ LME6 clear lids for above.
5. Adult cages (Figure 1.3).
6. Screw-top 118 mL plastic vials (e.g. Qorpak (Bridgeville, Pennsylvania, USA) # 3891, Figure 1.3).
7. Nonabsorbent screen cloth for small cages.
8. Large cotton balls.
9. Pupae collectors (Figure 1.3).
10. 10% solution of corn syrup or sucrose (v/v or w/v). Store at 4°C.
11. 90 mm filter paper disks (e.g. Whatman (Springfield Mill, Kent, England) #1003 090).
12. 2.5 cm wide filter paper rolls (e.g. Schleicher and Schuell (Keene, New Hampshire, USA) #08810).
13. 12-well spot plates for examination of larvae and pupae (e.g. Coors (Golden, Colorado USA) #60427).
14. Rearing pans (Figure 1.1).
15. Sieves.
16. Featherweight forceps (e.g. Bioquip (Gardena, California, USA) #4748, Figure 1.3).
17. Aspirator materials (Figure 1.3).
18. Egg-washing apparatus (Figure 1.3).
19. Household bleach.
20. Compressed CO_2, regulator, tubing for anaesthesia (Note 6).

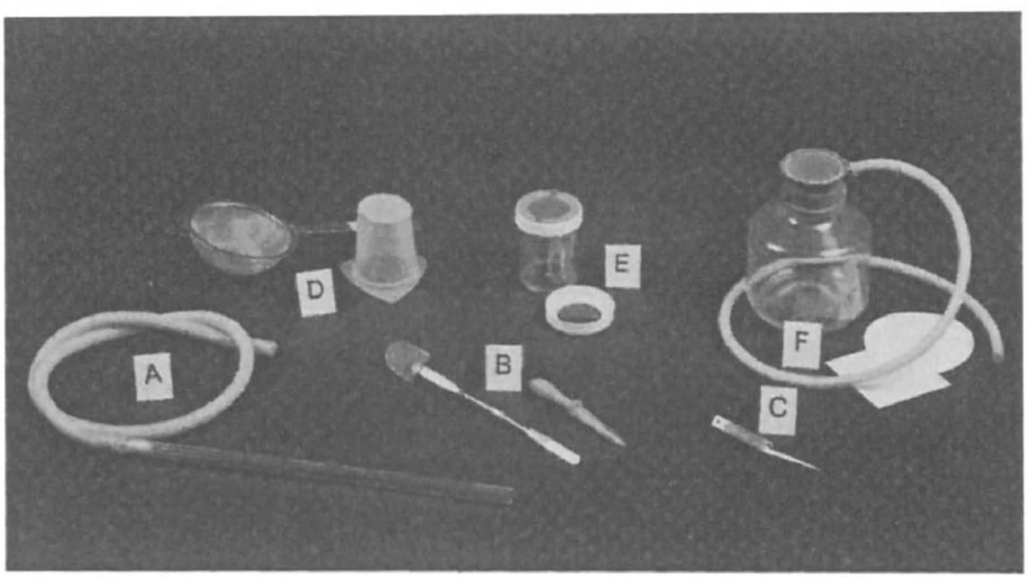


Figure 1.3 Miscellaneous mosquito handling equipment. (A) Adult mouth aspirator constructed of rigid plastic and latex tubing. A small insert consisting of a square of fine screen cloth wrapped around a coil of film in the plastic tube prevents adults from being inhaled. (B) Scoop and dropper-type pupa collectors improvised from common laboratory equipment. (C) Featherweight forceps are delicate enough that adults can be lifted by the tarsi without damage. (D) A stainless steel food sieve for straining fourth instar larvae and pupae, and a disposable beaker sieve with a fine screen glued in the bottom to retain young larvae. (E) Transparent 118 mL vials used for collecting eggs from single females and single-pair matings. The top has an opening covered with overlapping screen cloth through which adults can be aspirated. The vial is typically lined with filter paper and filled half-way with water for oviposition. A sugar-water pad can be laid on top for long-term adult maintenance. (F) Vacuum egg-washing device made by breaking off the top chamber of a 500 mL disposable filtration device and removing the filter membrane. Filter paper squares or disks are placed on the filter support and vacuum is applied *via* the normal outlet.

1.3 METHODS

1.3.1 Large-scale method

1. Day 1. Blood-feed adult females (Note 7) until satiated (~10 min). *An. gambiae* and *An. stephensi*, particularly, should be fed until droplets of blood and urine beneath the mosquitoes are abundant. Inadequate blood-feeding will reduce the number of females laying and the number of eggs each lays. Adults should be 2–6 days old (Note 8). Place a well-saturated sugar-water source inside or on top of the cage if not already present. Large-volume sugar sources (>10 mL) should require no attention for one week, at which time they should be changed regardless of condition. To reduce weekend work during egg-laying and pupation, it is convenient to make day 1 Monday or Friday.
2. Day 2. No attention.
3. Day 3. No attention.
4. Day 4. Line a clear cup with 9 cm long strips of filter paper and add water until the paper is half-covered. Introduce the cup into the cage.
5. Day 5. Remove the egg-laying cup. Remove dead adults from the egg-laying cup with forceps. Surface sterilization to reduce microsporidia infections may be achieved by washing the eggs onto filter paper on the vacuum apparatus (Figure 1.3) and soaking for 30 s in 1% household bleach. Follow with a water rinse to remove the bleach. Place a pan of water for each strain directly onto the shelf, and carefully wash eggs into pans for hatching. If the pans are not disturbed, no filter paper liner is necessary to keep the eggs moist. However, do not allow the eggs to dry out by becoming stranded on the side of the pan.
6. Day 6. Feed hatching larvae approximately 1.5 mL of food per 400 mL water.
7. Day 7. No attention.
8. Day 8. Divide the larvae into additional containers or discard excess larvae. Do not reuse the hatching pan unless you wipe it with a sponge and rinse it with hot water. This helps keep microbial growth under control.
9. Days 9–13. Continue thinning and feeding larvae as needed (Note 9 and Table 1.1 regarding feeding rates).
10. Days 14–18. Pupae develop and must be collected. *An. gambiae* (and most *Anopheles*) pupae must be collected daily and placed in clean water. Generally, pupae are scooped up with a small net, or individually pipetted out of the larval container (Figure 1.3, items in B). For large numbers of pupae, all immatures can be strained out of the rearing water and placed in ice water. Pupae will float and can be strained off, and the larvae, which will sink, can be returned to the rearing water. Exposure to ice water should be as brief as possible to avoid mortality. Place the pupa cup into the adult cage, making sure that a sugar-water source is provided since adults will die unless sugar water is supplied within about 24 h after emergence. On the first day of pupation, *Anopheles* adults will consist of a larger proportion of males than females so be sure sufficient females are present before discarding excess larvae (Note 10 for mating suggestions).

1.3.2 Small-scale method

1. Day 1. Blood-feed adult females (Section 1.3.1, step 1 and Note 7) until satiated. Place a well-saturated sugar-water pad on top of the cage, and cover the cup

Table 1.1 Recommended feeding rates for mosquito larvae

		Days after hatching								*Pupation*	
	Hatch	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
Large scale											
No. larvae	1500	1500	600	450	300	300	300	250	150	50	15
Water (mL)	800	800	800	800	800	800	800	800	800	800	800
Food (mL)	2–3	0	3	1.5	3	6	8	12	12	8	3
Small scale (e.g. iso-female)											
No. larvae	100	100	100	100	100	100	100	75	50	25	5
Water (mL)	50	100	100	300	300	300	300	300	300	300	300
Food (mL)	2 drops	0	0.5	0	0.5	1	2	4	4	1	0

with an inverted clear lid. This will keep the sugar-pad moist for 2 days. These small cages (Figure 1.2E) will hold up to 100 adults, a number adequate for stock maintenance. Overcrowding adults in these cups will reduce longevity greatly. To reduce weekend work during egg-laying and pupation, it is convenient to make day 1 Monday or Friday.

2. Day 2. No attention.
3. Day 3. Wet sugar pads if necessary.
4. Day 4. Line a 118 mL clear vial with 9 cm long strips of filter paper, and add water until the vial is half-full and the paper is half-covered. Cap the vial and aspirate a single gravid female into the vial. Avoid smaller vials since larval crowding and death will result. (Alternatively, eggs may be collected from the small cages *en masse* by placing an egg-laying cup covered by a lid with a hole under the adult paper cup; Figure 1.2F).
5. Day 5. Remove the females and return to cage. Remove any dead females from the vials.
6. Day 6. Feed hatching larvae two drops of larval food.
7. Day 7. Count hatching rate (optional) and transfer larvae to a clear plastic cup or larger tray. Feed 0.25–0.5 mL.
8. Day 8. No feeding.
9. Days 9–13. Feed as needed. Transfer to larger trays as they develop (Note 9 and Table 1.1 for feeding details).
10. Days 14–18. Collect pupae (Section 1.3.1, step 10). Place in fresh water in a clear cup for emergence. Cover with a lid with a hole in the centre, and place adult cage on top. Provide a sugar source.

NOTES

1. Maintaining appropriate humidity can be accomplished simply by introducing cups of water with sponge wicks into the adult holding chamber, or by more elaborate, but troublesome, humidifiers. By keeping the adult chamber sealed, passive humidifying methods are adequate.
2. Sterilization of larval containers and adult cages is desirable if only to ensure that no eggs or larvae contaminate a stock. However, this measure is not essential if materials are kept clean and dry between use. Autoclaving is desirable, but for containers that cannot be autoclaved, household bleach is effective.
3. Gravid females of other strains, and especially species such as *An. stephensi*, will lay eggs in open containers. To reduce this possibility, we recommend covering larval culture containers. This measure also maintains proper larval water temperature since the water temperature will be slightly below the air temperature due to evaporative cooling.
4. The TetraMin Baby-E fish food we recommend is neither the least expensive, nor the most widely available food that can be used for mosquito rearing. However, we have found that it is of predictably high quality, and even inexperienced persons obtain good results with various species (including *Aedes*). Moreover, the expense is small relative to other modern laboratory equipment and reagents. We have experimented with TetraMin Adult Flake food ground by various methods and Baby-L. Neither of these yields the good results of

Baby-E. A variety of foods that have been used with at least some success are discussed by Asahina (1964), but yeast is a recommended component found in most recipes.

5. Items 2–4 in Section 1.2 are specified because their dimensions are similar, producing a convenient modular design. Similar units can be constructed from various commercially available containers.
6. Adults can be anaesthetized easily with CO_2 pooled in a shallow (3 cm) tray. A CO_2 stream bubbled through water, and running into a tray through a diffuser is convenient for anaesthetizing mosquitoes while working under a microscope and routine sexing. The CO_2 concentration should be as low as effective, and exposure limited to less than 10 min. Alternatively, brief exposure in a freezer will immobilize adults reversibly. However, such exposure must be closely monitored to prevent mortality.
7. Anaesthetized rabbits and guinea pigs, and artificially preserved blood-meals (e.g. Bailey, 1978) can be used. Though artificially preserved blood-meals seem attractive, long-term successes maintaining anything but very large colonies by this method are rare. For animal care procedures, readers should conform to local, state, and national guidelines such as the *U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training* and the *Guide for the Care and Use of Laboratory Animals* (Office for Protection from Research Risks, National Institutes of Health, Bethesda, MD 20892).
8. Anopheline mosquitoes are generally kept in culture continuously since no methods are available for long-term storage of eggs to induce adult diapause, or for cryopreservation of embryos (but see Bailey *et al.*, 1979). Therefore failure of one generation to develop can lead to loss of a strain unless back-up material is kept on hand. A wise strategy is to maintain adults at least until their progeny have oviposited. We do not culture multiple cohorts of a generation unless we experience rearing failure.
9. Two water characteristics indicate the amount of food that should be fed: odour and turbidity. When the cover is removed from a larval container, the odour should be organic, but not offensively foul. A strong unpleasant odour indicates too much food has been added previously, and little, if any, food should be given until the odour is reduced or a water change is made. Turbidity – in contrast to a yellow colour – indicates that a large amount of suspended matter is present, and the feeding rate should be reduced. Insufficient feeding is indicated by larval consumption of faeces leading to long cylindrical faecal matter on the bottom of the container (Table 1.1 for approximate feeding rates).
10. Genetic studies are often limited by the inability of small numbers of individuals to mate successfully. We find that mating among more than approximately 10 males and females will occur readily after 24 h in either small paper cups, or in any of the larger cages. One male will mate with several females; however, females are generally monogamous. Virgin females can be collected within 24 h of emergence, or isolated as pupae based on their genital lobes being shorter than those of males. We have successfully obtained single-pair matings by introducing a newly emerged male and 1–3 females into transparent vials for isofemale egg-laying for 7 days. Success seems to be somewhat dependent on a sunset light cycle. Alternatively, a more successful strategy for single-pair matings is to place two phenotypically distinct males into an egg-laying vial with 1–3 females, or into a small cage with 5–10 females. If progeny are collected by indi-

vidual egg-laying, parentage can easily be determined. In extreme conditions, assisted copulation can be performed (Baker *et al.*, 1962) although this is very time consuming and fertility of the resulting matings is not as high as after free mating. Nevertheless, numerous anopheline colonies have been maintained continuously by assisted copulation including *An. dirus* (W. Collins, pers. comm.), *An. superpictus, An. labranchiae, An. sacharovi* (Coluzzi, 1964), *An. punctipennis* and *An. maculatus* (Ow Yang *et al.*, 1963) (for additional details on this method, see Chapter 8, Note 1).

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CHAPTER TWO

Care and maintenance of Aedes *mosquito colonies*

Leonard E. Munstermann

2.1 INTRODUCTION

Nearly 1200 species of *Aedes* have been described and given a formal Latin name. These species can be found in diverse habitats from Polynesian islands and African deserts to ice pools at the Arctic Circle. The larval nutrition and adult female host preference for blood-feeding is equally variable. Mating behaviours and preference for oviposition substrate are also distinctive for a given species. Considering first, the number of species and then, the diversity in habitat, nutrition and behaviour among these species, a single, simple rearing method cannot be devised that will be successful for all of them. In a recent manual of mosquito rearing, 59 species of *Aedes* are listed with methods for laboratory rearing (Gerberg *et al.*, 1994). This listing includes species that are difficult or impossible to maintain in continuous colony. This is undoubtedly a very conservative estimate of the species that have been colonized but, even so, it indicates that far less than 10% of the described species have been successfully colonized.

Certainly, many additional species have been subjected to exhaustive (and unpublished) attempts to rear and colonize, but have met with complete failure. In fact, a number of the species in the Gerberg *et al.* (1994) list can be reared only from field-collected eggs or maintained by induced mating procedures (for methods, see Munstermann and Wasmuth, 1986b). Some species, such as *Ae. vexans* are represented in colony by a single highly selected strain; others such as the African *Ae. simpsoni* are colonizable only from specific portions of its range. Therefore, even for those colonizations reported as successful, a great amount of variation is seen in (i) methods of colonization, (ii) number of generations completed in isolation from the field and (iii) strains from different geographic areas. Finally, rearing of insects can depend on a person's rearing intuition or quality of laboratory environment. Even when rearing methods have been carefully detailed, successful maintenance in one setting may invariably fail in other settings.

Here, a protocol will be proffered that is simple and sufficient to rear many species in the *Stegomyia* subgenus, including *Aedes aegypti* (Munstermann and Wasmuth, 1986a). This latter is the most widely used *Aedes* species in colony, and it is among the least troublesome to rear. If attempts to rear other species fail while following the

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aegypti protocols, clues to the problem may be found in the following descriptions of colony use and life cycle.

2.1.1 Uses of a mosquito colony

The method of colony maintenance may differ depending on the purpose the colony may serve (Munstermann, 1994a). If maintenance of field traits or broad range of genetic variability is desired, routine incorporation of new field genomes into the colony is recommended. Strong selection pressures occur in the course of colonization and during routine colony maintenance. Alternatively, if the goal is to use the colony as a homozygous reference standard or source of genetically homogeneous DNA, then a careful regime of brother–sister inbreeding may be required. Note that high rates of polymorphism can be maintained in the course of laboratory adaptation due to the development of balancer chromosomes or reduction of recombination.

For many purposes, whole organism DNA may not be required. In these cases, development of tissue culture provide an undifferentiated 'colony' that may be much easier to maintain and store than the whole organism (Munstermann, 1994b).

2.1.2 The mosquito life cycle

Completion of the life cycle in the laboratory involves special care at each of the four stages: egg, larva, pupa and adult. In the following, each stage will be described briefly, along with the most important characteristics and requirements. A few of the common, stage-specific rearing problems will be indicated as they relate to the most sensitive requirements.

(a) The egg

The eggs of *Aedes* mosquitoes are approximately 1 mm long and 0.25 mm in diameter. The hard black shell provides the egg with desiccation resistance and is often sculptured in species-specific patterns. The desiccation resistance is a distinct advantage to storage of the species for longer periods but can provide disappointment for the unwary. The eggs of *Aedes aegypti* have a half-life of up to a year if maintained in humid conditions, but the closely related *Ae. albopictus* have a survival rate of less than half that time. Other species such as *Ae. vexans* and other univoltine species are said to survive 2 years or more. The eggs of some species require a specific photoperiod for hatching; others require a period of cold. Often each of these characters is related to the native habitat of the species, e.g., species in dry environments are more likely to be highly desiccation-resistant; those from the tropics are unlikely to require special photoperiod regimes or cold treatment.

There are two stimuli for hatching. Immersion in water alone is often sufficient. Low oxygen tension seems to speed the process. Boiled water, vacuum or bacteria (associated with larval food) are often used to stimulate a more synchronous hatch.

(b) The larva

The larvae are strictly aquatic. If the habitat dries, the larvae die. The larva goes through four successive moults to reach the pupa stage. The larva grows from approximately 1.5 mm to 15 mm long after the third moult. Maximum sizes can vary

from less than 10 mm to more than 20 mm depending on nutrition and species. Under optimum conditions, the larva stage can be as short as 4 days; under starvation conditions or suboptimal temperature regimes, they may remain as larvae for months in a diapause state. Larval food consists of bits of organic debris, bacteria, fungi and algae that are collected from the bottom of the aquatic habitat by means of specialized mouth appendages.

(c) The pupa

After the fourth larval moult, the comma-shaped pupa emerges. It, too, is strictly aquatic although generally it can survive exposed to air on moist surfaces. The pupa does not take any nutrition, even though it may be very active in the aquatic milieu. The pupal stage permits the insect to undergo the extraordinary structural and metabolic changes that will permit the emerging adult to survive in a terrestrial environment. Musculature, wings, scales, mouthparts and other internal and surface structural elements must be formed before this stage is complete. This period lasts 2–3 days, then the pupa floats to the air–water interface and the adult emerges onto the water surface.

(d) The adult

In the first 24–48 h of adult life, internal development continues to complete the transformation to terrestrial life. The main external manifestation of these changes is the complete rotation of the male genitalia (dististyles changing from antero-dorsal to antero-ventral). After the genitalia rotation is complete, mating becomes possible. A single mating results in sufficient sperm in the female spermathecae (three sclerotized sperm storage structures) to last a lifetime. Females become capable of blood-feeding 2–4 days after emergence. Carbohydrate food sources of sucrose or simple sugars are the required adult maintenance nutrition. Low humidity and lack of carbohydrate source can drastically shorten adult life expectancy.

In most mosquito species, blood is required by the female for egg development. Some mosquitoes are very specific in host selection, others will feed on a broad spectrum of hosts. After blood-feeding, the eggs take 3–5 days to develop before oviposition can occur. Selection of oviposition substrate varies – *Ae. vexans* requires soil, tree hole species prefer grooved wood; others may oviposit directly onto a water surface.

2.2 MATERIALS

Most of the materials described below are common items that can be purchased locally or through scientific supply houses.

2.2.1 The egg

1. Paper towelling.
2. Pipette.
3. Plastic storage box.
4. Shallow hatching pans, flat bottom preferred.
5. Water.

6. Potassium chlorate.
7. Beaker, plastic or glass.
8. 15°C incubator.
9. N_2 gas or sealable jars for boiled water.
10. Larval food (see below).

2.2.2 The larva

1. Rearing trays: Flat bottom are preferred due to the bottom feeding behaviour of the larvae.
2. Plexiglass tray cover.
3. Conditioned tap water: Larvae may be susceptible to water treatment chemicals; it is preferable to distil water or to 'condition' the water by allowing it to stand overnight.
4. Pipettes: 5 mL plastic accordion transfer pipettes.
5. Larval food: A simple food is rabbit or hamster food. Some researchers prefer pulverized fish food. A more complex, but more nutritious and semi-quantitative, method is the use of a liver powder suspension. Sixty grams of liver powder is added to 1 L of hot (60°C) tap water and shaken vigorously; it may be stored at 0°C for up to one month.
6. Temperature/light controlled insectary or incubator.
7. Liver powder (ICN Pharmaceuticals).

2.2.3 The pupae

1. Pipettes: 5 mL plastic accordion transfer pipettes.
2. Cage for adults.
3. Tea strainer.
4. Cellucotton balls.
5. Pupa separator.

2.2.4 The adult

1. Adult cages: Adult cages are available commercially (Gerberg *et al.*, 1994; Munstermann and Wasmuth, 1986a, 1986b) or inexpensively constructed (Munstermann and Wasmuth, 1986a, 1986b). They can be constructed from plastic or paper cartons. A net top is held in place by rim of original cover (top cut out) or rubber bands. An opening cut into side of container permits removal of adults or oviposition media. A cloth sleeve stapled to this opening will forestall adult escapes. Details of adult cage construction may be seen in Munstermann and Wasmuth (1986a).
2. Sucrose solution, 10% in water.
3. Cellucotton balls.
4. Aspirator: A simple aspirator can be made with a 30 cm length of plastic tube (i.d. 10 mm) with one end covered with a fine-mesh screen and attached to 50 cm (1 cm diam) rubber tube.

5. Mice: Laboratory white mice for feeding adult female mosquitoes for egg production.
6. Sodium pentabarbitol (Nembutal™).
7. Oviposition cup: A small paper cup lined with paper towelling.
8. Paper.

2.3 METHODS

Note again that the methods described herein apply to *Ae. aegypti* and additional species listed in Munstermann and Wasmuth (1986a). Special methods required by other species may be described in Gerberg *et al.* (1994) or Munstermann and Wasmuth (1986b).

2.3.1 The egg

(a) Egg collection

1. Remove the oviposition substrate from the water container and allow the eggs to dry slowly at insectary temperatures.
2. The eggs require 4 days for completion of embryonic development; during this period, they will not hatch and are susceptible to low humidities.
3. The eggs are lightly glued by the mosquito during oviposition to the paper, cloth or wood substrate; eggs oviposited on water surface may be pipetted to the surface of filter paper.

(b) Egg storage

1. Place in plastic storage container with a saturated solution of potassium chlorate and store at 15°C. A saturated solution of $KClO_3$ maintains the humidity at an optimum 85%, i.e., not too wet to induce hatching and not too dry to promote excessive desiccation.
2. Storage at other temperatures may be convenient, but shortens the egg half-life.

(c) Egg hatching

1. Immerse oviposition paper with eggs in deoxygenated water.
2. Add a small amount of larval food. Water can be deoxygenated by bubbling nitrogen gas through it for several minutes, by boiling, cooling and sealing the water, or by 'ripening' the water by adding the larval food several hours before hatching the eggs.
3. Select a number of eggs such that will produce approximately 300 or fewer larvae per litre of fluid environment.
4. *Ae. aegypti* normally oviposits readily on white paper towelling; the eggs are easily handled and shipped in this manner.

2.3.2 The larva

(a) Aquatic environment

1. The hatched larvae (300) are placed in a 1–2 L container with 1 L of tap water at an optimum insectary temperature. Optimum temperatures vary with species – for *Ae. aegypti*, 26°C; for many temperate *Aedes*, 21°C or lower.

2. After hatching, add a small amount of larval food, either a single pellet of rabbit or hamster food or a pinch of pulverized fish food. If liver powder is preferred, before each use the suspension should be shaken vigorously; 5 mL are added to the rearing pan as needed – approximately once per day until pupation.
3. Place a transparent tray cover over each rearing pan. This practice will: (i) prevent evaporation and lowering of temperature; (ii) forestall the introduction of air-borne pathogens or other mosquito larvae – by accident or oviposition; (iii) prevent escape of adult mosquitoes if pupae were not removed in a timely fashion.

2.3.3 The pupa

1. Remove pupa from larval environment on a daily basis, thus preventing adult emergence in larval trays. Five mL accordion transfer pipettes are excellent tools for the purpose; cut off the pipette tips to permit easy entry of pupae. A tea strainer is also useful for concentrating pupae and placing them in clean water.
2. Place pupae for emergence in desired cage; 50 per 0.5 L cage, 3–500 per 4 L cage. Pupae may be placed on moist paper towelling supported by water-saturated cellulose cotton balls; this prevents weaker adults from dying on the water surface.
3. Separation of sexes for genetic studies is best done at pupa stage. The sexing of most aedine pupae can be 95–99% accurate by size determination alone, if the larva rearing conditions are good. The larger and later pupating individuals are females; the smaller and early pupating individuals are males. If 100% accuracy is required, all individuals must be examined at 20× with a dissection microscope – the males are recognized by large lobed structures protruding beneath the pupal paddles at the terminus of the abdomen.

2.3.4 The adult

1. The adults are allowed to emerge into the adult cages or transferred from emergence cages by aspirator.
2. A cellucotton pad moistened with 10% sucrose placed on the cage top or within the cage will provide a sufficient energy source for both males and females. Raisins, sugar cubes, fruit slices and cellucotton saturated with honey may also be used as carbohydrate sources. Replace as needed, particularly if mouldy or dried.
3. For blood-feeding, anaesthetize one white mouse for 100 female mosquitoes and place it on the netting or screen at the cage top. A dose of 0.25 mL of Nembutal™ suffices to anaesthetize a mouse for 10–20 min. All females may not feed initially; one feeding will produce 80–125 eggs/female. Females may be blood-fed repeatedly every 4 days. Eight or more oviposition cycles can be obtained.
4. Place an oviposition cup inside the adult cage. This provides water for both sexes and an oviposition surface for the female at the water–air interface. The cup should be changed every 3–4 days – oviposition begins 4 days after blood-feeding. If eggs are present, treat paper as described under Section 2.3.1(a).

NOTES

Colonization and maintenance of more difficult species can require specialized equipment and techniques. The following is only a sampling of solutions to specific problems.

Special insectary needs

1. An insectary where temperature, humidity and photoperiod can be controlled is very important for setting an environment appropriate to the species. These three factors can have an effect on all mosquito stages.
2. A dawn–dusk timer device that gradually lowers the lighting in the 'evening' and increases in the 'morning' can contribute to the mating success of many species, particularly those species that mate in swarms.
3. Tall cages may be necessary for mosquitoes that mate in swarms or require more mating space.
4. Negative air pressure and triple-screened rooms may be necessary for laboratories using infected mosquitoes. These and other recommended details can be found in Higgs and Beaty (1996).
5. An induced mating setup may be required in instances where the males and females refuse to copulate; nitrogen gas (mosquito anaesthesia), minuten pins and assemblage are described by Munstermann and Wasmuth (1986b).

Problems in colony establishment/maintenance

The egg stage

1. Eggs dry and die at low humidity conditions.
2. Asynchronous hatch can be due to insufficient reduction of oxygen tension, older, slightly desiccated eggs or genetically controlled 'instalment hatching'.
3. Contamination of strains or species can easily occur by egg transfer on fingers, pipettes or containers.
4. Shipping eggs by air, or in winter months, can result in complete mortality for cold-intolerant eggs. Always ship eggs in insulated bags.

The larva stage

1. Overfeeding produces 'scum' on water surface, preventing larval breathing – certain foods are more prone to scum production than others. If the problem is recurrent, a fish tank aerator provides sufficient turbulence to prevent scum.
2. Crowding produces very small mosquitoes, mortality and cohort asynchrony.
3. Contamination of rearing pans with fungus, bacteria or microsporidia can result in very high larval mortality.

The pupal stage

1. Malnourished larvae produce pupal mortality and pupal sizes that overlap for the sexes.

2. If pupae are not removed to adult cages *every day*, adult emergence will occur within larval rearing pans and can result in escaped mosquitoes
3. Pupal mortality is often increased unless pupae are removed to clean water.

The adult stage

1. High temperature, low humidity kills mosquitoes before mating or blood-feeding.
2. Insufficient nutrient source and lack of water considerably shortens adult longevity.
3. Cage size or other conditions may prevent mating.
4. Failure of blood-feeding may result from attempting to feed females that are either too young or too weak. Alternatively, a different host may be needed for feeding the mosquitoes (i.e. rabbit, chicken, etc.)
5. Failure of oviposition may result when an inappropriate oviposition substrate is provided.

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CHAPTER THREE

Care and maintenance of phlebotomine sandfly colonies

Govind B. Modi

3.1 INTRODUCTION

Phlebotomine sandflies include about 700 species and belong to the order Diptera, family Psychodidae, and subfamily Phlebotominae. They are important vectors of leishmaniases, arboviruses (sandfly fever and other) and bartonellosis (Carrion's disease). About 70 species of sandflies are proven or suspected vectors of these diseases (Lane, 1993). Sandfly transmitted diseases are widely distributed in tropical and subtropical countries, extending through Central and South America, Central and Southeast Asia, India, China, the Mediterranean Basin, and Africa. Several autochthonous human cases of cutaneous leishmaniasis have been discovered in Texas, dispelling the belief that sandfly-borne diseases do not occur in North America.

Laboratory colonies of sandflies are essential for studies on the systematics, biology, genetics, repellent/insecticide testing, vector potential, vaccine development and molecular approaches to blocking transmission of pathogens. Since the first successful colonization of sandflies in 1907 by Grassi (Ward, 1977), a variety of techniques have been described for rearing these insects in the laboratory (bibliography by Killick-Kendrick *et al.*, 1991). Generally, however, sandflies have a reputation for being difficult and labour-intensive to breed. Although impressive progress has been achieved in rearing technology for these insects in the past decade, there are very few laboratories in the world where colonies of these flies are currently maintained. For example, in the USA, the Walter Reed Army Institute of Research, Washington DC, and Yale University, New Haven, Connecticut, are the only two institutions currently maintaining laboratory colonies of both New and Old World phlebotomine vectors (Table 3.1). Of about 45 species of sandflies which have been colonized in the laboratory so far, only a few, i.e., *Phlebotomus argentipes, P. ariasi, P. duboscqi, P. langeroni, P. longipes, P. martini, P. orientalis, P. papatasi, P. pedifer, P. perfiliewi, P. perniciosus, Sergentomyia africana, S. schwetzi, Lutzomyia anthophora, L. diabolica, L. flaviscutellata, L. gomezi, L. longipalpis, L. sanguinaria, L. trapidoi* and *L. walkeri* have been successfully reared in large numbers (Beach *et al.*, 1983, 1986; Chaniotis, 1986; Endris *et al.*, 1982; Gemetchu, 1976, 1977; Johnson and Hertig, 1961; Killick-Kendrick and Killick–Kendrick, 1987; Killick-

Molecular Biology of Insect Disease Vectors: A methods manual. Edited by J.M. Crampton, C.B. Beard and C. Louis. Published in 1997 by Chapman & Hall. ISBN 0 412 73660 8.

Kendrick *et al.*, 1977; Morales *et al.*, 1984; Maroli, 1983; Maroli *et al.*, 1987; Ward, 1977) and about half a dozen of these have been mass colonized (Modi and Tesh, 1983; Lawyer *et al.*, 1991).

This chapter describes a technique for collection and transportation of wild sandflies for establishment of a laboratory colony (simplified from Killick-Kendrick, 1987; Killick-Kendrick and Killick-Kendrick, 1991) as well as maintenance of these insects in the laboratory to provide sufficient material for experimental work. The approach described here for maintenance of productive laboratory colonies is a combination of techniques used for mass culture of these insects over a decade (Endris *et al.*, 1982; Modi and Tesh, 1983; Lawyer *et al.*, 1991). While describing the techniques presently used (a sophisticated setup), an attempt has been made to keep in mind unavailability of certain expensive equipment in developing countries, for which alternatives are given (see Notes). Having spent a number years in the establishment and maintenance of several colonies of these insects in both developing and developed countries, the author is of the opinion that in the absence of sophisticated equipment (such as automated temperature, humidity and light controlled incubators or vacuum-operated aspirators), laboratory colonies of sandflies can be established and maintained using alternative techniques, imagination and commitment.

3.2 MATERIALS

3.2.1 Collection and transportation of wild sandflies

1. Aspirators: Mouth and/or battery operated (made by hand or purchased from Hausherr's Machine Works, Toms River, NJ 08753, USA).
2. Light traps: CDC miniature light traps (Hausherr's Machine Works).

Table 3.1 List of sandfly species in laboratory colony*

Species	*Origin*	*Medical importance*
Phlebotomus argentipes	India†‡	*Leishmania donovani*
	Nepal†	
Phlebotomus duboscqi	Kenya†‡	*Leishmania major*
Phlebotomus halepensis	Jordan†	
Phlebotomus sergeti	Pakistan†	*Leishmania tropica*
Phlebotomus papatasi	India‡	*Leishmania major*
	Israel†	
	Saudi Arabia†	
Sergentomyia schwetzi	Kenya†	
Lutzomyia longipalpis	Brazil†‡	*Leishmania chagasi*
	Columbia‡	
Lutzomyia shannoni	Columbia‡	

* Colonies currently maintained in the USA.
† Walter Reed Army Institute of Research, Washington DC, USA.
‡ Yale University, New Haven, Connecticut, USA.

3. Adult holding containers: Nylon cloth cages (18–20 cm^3) supported with a plastic or steel frame (25 cm^3); plastic (polymethyl-pentene) jars 125 mL capacity (Nalge Co., Rochester, NY 14602, USA); and plastic snap-top vials 7 dram (Fisher Scientific Co., Pittsburgh, PA 15219, USA). Prior to use, a 2 cm thick layer of plaster of Paris is poured into the jars and vials and the plaster is allowed to dry for 2–4 days.
4. Flash lights.
5. Transporting boxes: Polystyrene (Styrofoam) or plastic rectangular boxes with tight fitting lids.
6. Other supplies: Cotton balls, corn syrup (Karo) (Best Foods Division, CPC International Inc., Englewood Cliffs, NJ 07632, USA), rubber bands, sponges, adhesive tape (various colours).

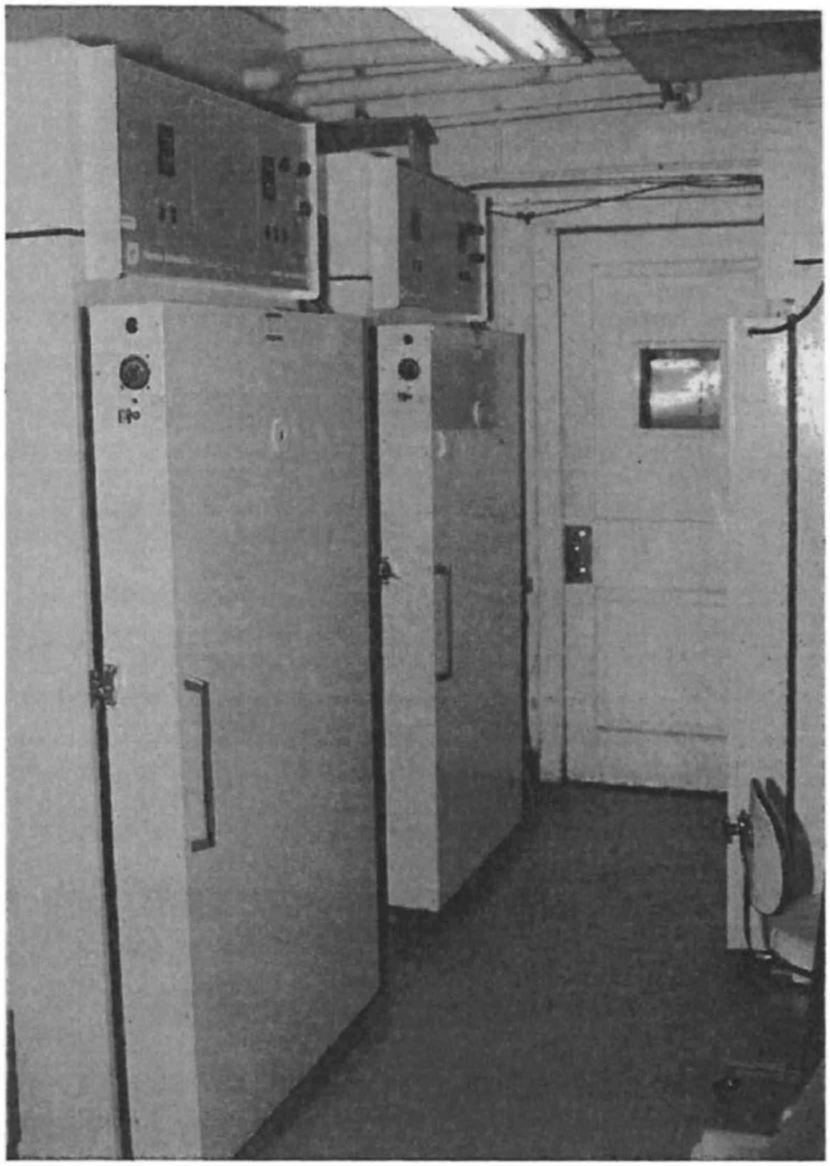


Figure 3.1 Reach-in incubators for holding adult cages and rearing jars.

3.2.2 Laboratory rearing

1. Incubators: Reach-In incubators (Figure 3.1) with humidity, temperature and light controls (Forma Scientific Inc., Marietta, Ohio 45750, USA).
2. Oviposition/rearing jars: Plastic (polymethyl-pentene) jars 500 mL and 125 mL capacity (Nalge Co.). Prior to use, 30–35 holes (7.0 mm in diam.) are drilled in the bottom of each jar (about 10 holes in a 125 mL jar) and a layer of plaster of Paris (2 cm thick) is poured into the upright jar. The plaster surface is allowed to dry for 2–4 days (Figure 3.2).
3. Adult holding/feeding cages: Made from a modified glass aquarium (51 × 32 × 27 cm or 31 × 31 × 31 cm). The bottom and back side of the aquarium is covered with 1 cm layer of plaster of Paris. The front of the aquarium is made of a 6 mm thick acrylic plastic (plexiglass) with a centre opening (15 × 15 cm) attached to the aquarium with silicone glue. A cloth sleeve (36 cm long × 15 cm wide) is secured to the front panel over the centre opening by compressing it between the panel and a removable plexiglass frame using brass screws and wing nuts (Figure 3.3).
4. Aspirator: Two types, a mouth aspirator, and a vacuum-powered aspirator, used to transfer females for oviposition from adult cages to oviposition jars. The vacuum-powered aspirator consists of an acrylic-plastic lid (12.5 cm diameter) which fits snugly on top of the oviposition jar. The lid has two ports, a larger funnel-shaped, which protrudes upward from the top of lid, and a smaller straight one, which protrudes downwards through the lid into a hole in the

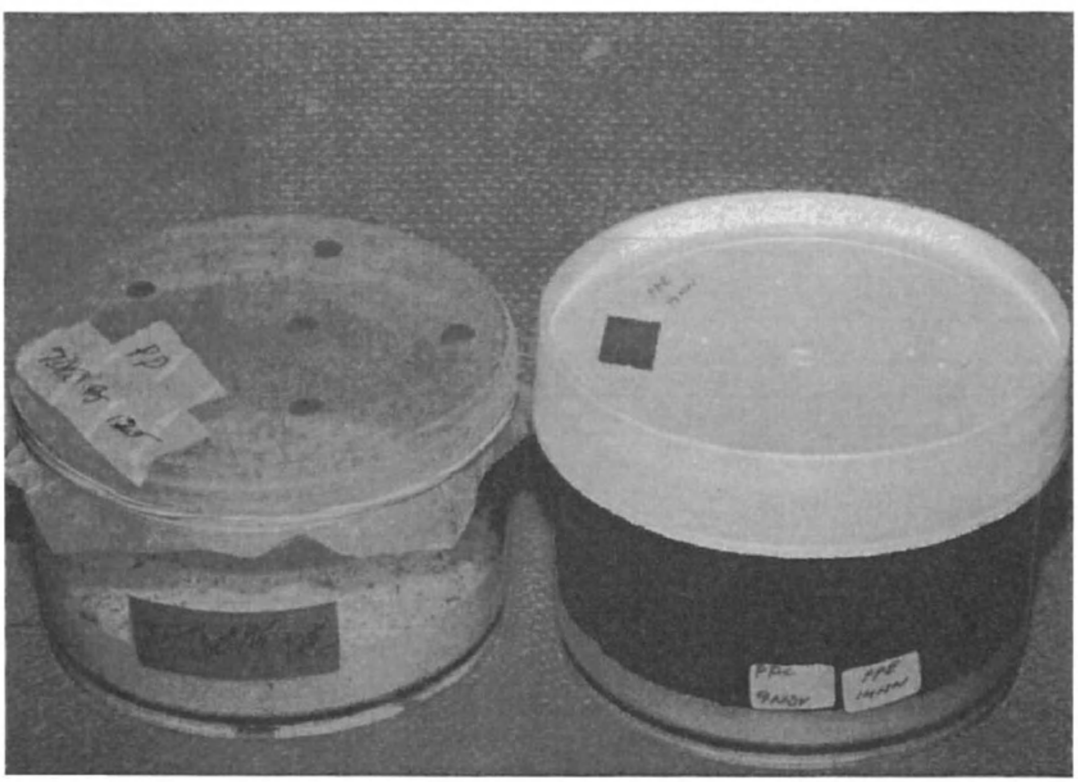


Figure 3.2 Oviposition/rearing jars.

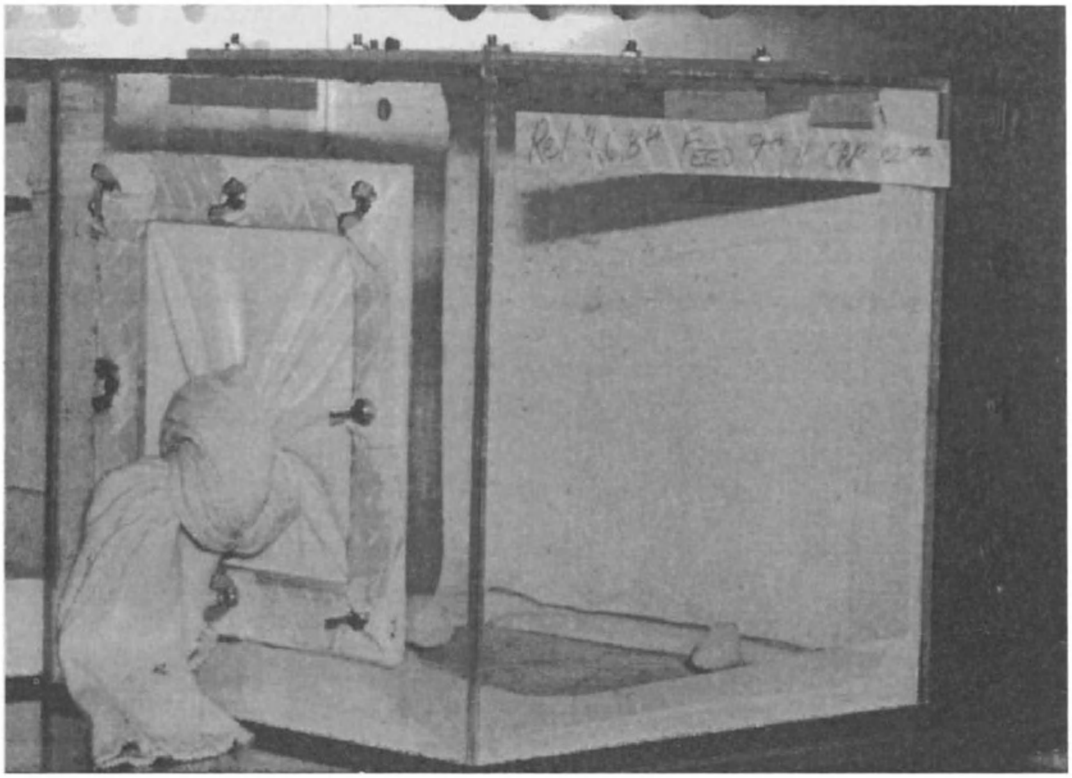


Figure 3.3 Adult holding/feeding cages.

nylon screen covering the oviposition jar to about 3 cm inside the interior of the jar. A flexible rubber tubing of about 1.5 cm diameter connects the funnel-shaped end to a laboratory vacuum pump (Gast Manufacturing Corporation, Benton Harbor, MI, USA). A smaller 1 m length of tubing of about 1 cm diameter is connected to the outer end of the intake port and a 10 mL plastic pipette is attached to the distal end of this tube.

5. Larval food: Mixture of dried rabbit faeces and rabbit food (Purina Rabbit Chow Complete Diet 5315, Ralston Purina Co., St. Louis, MO 63188, USA). Equal amounts (1:1 by vol or weight) of ground (in a portable mill grinder) dried rabbit faeces and food are mixed, spread in 1–2 cm thick layer inside plastic photographic trays (52 × 45 × 7 cm) saturated with water and incubated at RT in homemade acrylic-plastic cabinets (83 × 57 × 49 cm) for ageing. Each cabinet accommodates five trays on stainless-steel brackets mounted to the inner walls. A removable front panel fitted with tension latches and a foam rubber gasket provides a tight seal. The lower three inches of each cabinet forms a reservoir which is filled with water to keep the air inside the cabinet to near saturation. The cabinets are opened once a week to stir the moulding mixture in the trays, and to allow CO_2 and other undesirable gases to escape. The mixture after stirring is sprinkled with a small quantity of water each time (unless it is too wet) and the trays are put inside the cabinet again to continue ageing of the food. After about a month (when there is no apparent growth of mould), the trays are removed from the cabinets and kept in open air for 2–4 days to dry the food. The dried food is scraped from the trays and ground again in a mill grinder and stored in zipper-lock plastic bags at –20°C until use. The food which is in use is

kept in a plastic box in a refrigerator after each use to avoid contamination with mites and/or fungus.

6. Animals for feeding adults: Hamsters; and anaesthesia for hamsters (ketamine 10 mg + xylazine 2 mg/100 g body weight).
7. Plastic trays: Plastic photographic trays (40 × 34 × 6.5 cm) for holding oviposition jars.
8. Other supplies: Apples (for adult flies), corn syrup (Section 3.2.1, step 6), cotton balls, rubber bands, nylon cloth, coloured adhesive tapes.

3.3 METHODS

3.3.1 Collection and transportation of sandflies

(a) Collection of sandflies in the field

Collection of the wild females mainly depends upon species (to be colonized) and its natural resting and feeding habitats. Commonly used methods for collecting females are: active catches of resting or feeding females from the walls of houses, human bait, animal dwellings/animal baits, caves, tree holes, and tree buttresses using a mouth or battery operated aspirator and a flash light; and/or CDC miniature light traps.

(b) Transportation of sandflies from the field

The blooded/gravid wild-caught females can be individually confined in small plastic snap-top vials (7 dram vials) or in groups of 20–25 (if they belong to the same species) in 125 mL plastic jars. The plaster layer of containers (vials and jars) should be moistened with water before transferring females for oviposition. The females should be provided with 50% corn syrup solution using either a cotton ball or drops placed on top of a small nylon screen glued to the container tops by drilling a small hole in the centre. These containers should be tightly placed in styrofoam or plastic boxes (with tight fitting lid) to avoid shaking during transport. A wet sponge should be placed in each box to provide additional humidity, and the boxes transported to the laboratory at a temperature between 20 and 30°C. Unengorged wild-caught females can be fed on a hamster, mouse, guinea pig, chick or rabbit. Human volunteers can be used if animals are not available, although it should be kept in mind that these females could be infected with pathogens. Animals need to be anaesthetized (Section 3.2.2, step 6) prior to sandfly feeding. After feeding, the females should be handled as referred to above for transportation to the laboratory. The females usually lay eggs on the plaster surface of the containers within a week.

3.3.2 Rearing in the laboratory

Sandfly colonies in the laboratory are maintained in Reach-In incubators at 26–27°C, 80–100% RH and 14:10 (L:D) photoperiod (Figure 3.1).

(a) Adults

Adult flies emerged in rearing jars (Figure 3.2) are released on alternate days (Monday, Wednesday and Friday) from the jars into adult holding/feeding cages (Figure 3.3) which are kept inside the incubators. Following the release, the flies are provided with fresh apple slices daily as a source of sugar.

(I) BLOOD-FEEDING

The females are offered two blood meals on hamsters usually 24 and 48 h after release in the adult cages. Each hamster is anaesthetized (Section 3. 2.2, step 6) and their abdomen shaved. The hamsters are placed on their back inside the adult cages. The animals usually remain asleep for 1–2 h, allowing sufficient time for hungry females to feed to repletion. Since the females prefer feeding in the dark, the lights inside the incubator doors are turned off during the feeding. The females usually feed on the shaved abdomen, ears, feet, nasal and tail area of hamsters. The engorged females (Figure 3.4) are left undisturbed in holding/feeding cages for 24–48 h after feeding for complete formation of peritrophic membrane, mating, diuresis and beginning of maturation of eggs. The females usually mate before, during and after the blood meal.

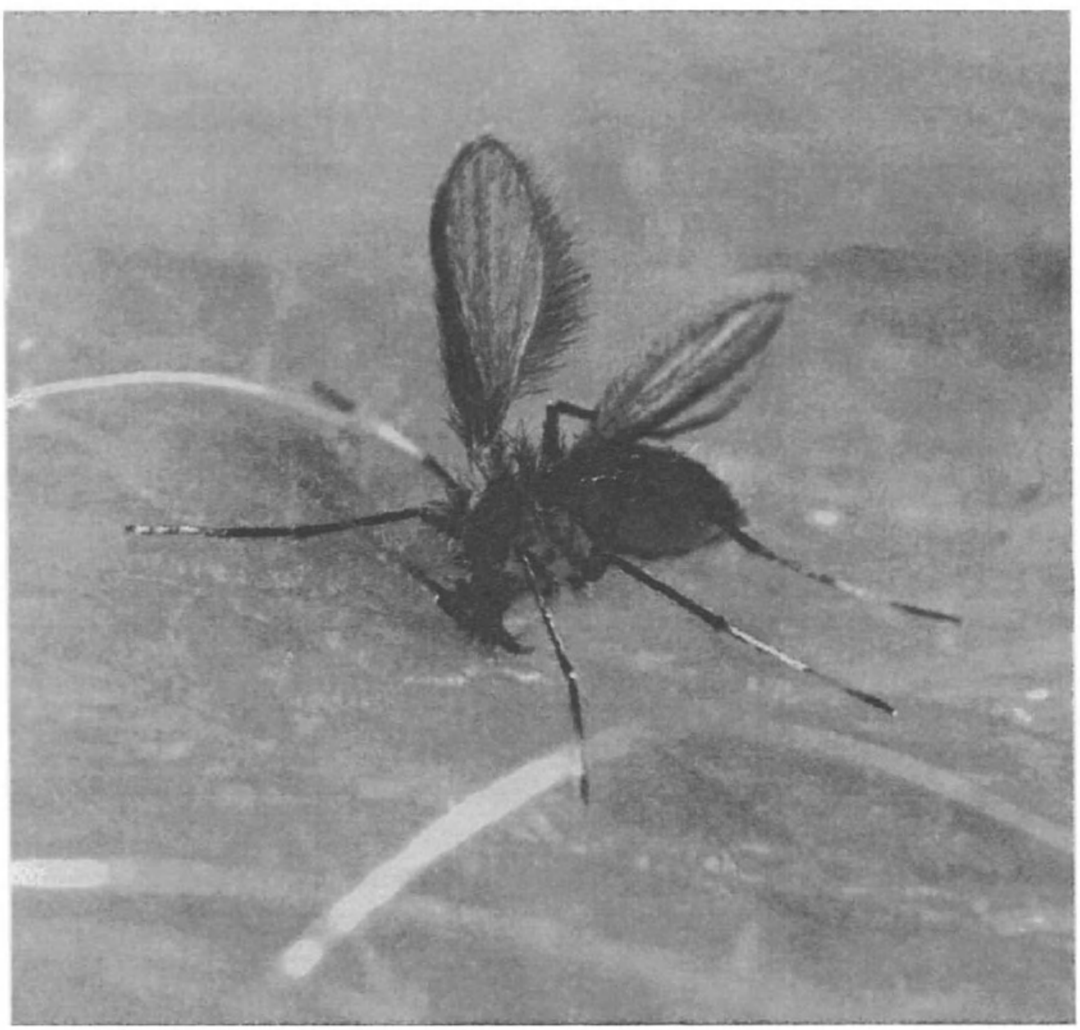


Figure 3.4 Engorged female of *P. papatasi*.

(II) OVIPOSITION

The engorged females are transferred to moist oviposition jars (Figure 3.2) 24 h after the second blood meal using a vacuum-powered aspirator (Section 3.2.2, step 4). To transfer females from adult holding/feeding cages to oviposition jars, the open end of the jar is covered with a nylon screen fastened with rubber-bands and a small hole is made in the screen to fit the inward protruding end of the plastic lid which is attached to the vacuum pump. The vacuum pump is switched on and adjusted to a low suction. The pipette end of tube is inserted in the adult cage and about 125 females are sucked into oviposition jars. After transfer, the hole in the nylon screen cover of the jar is closed using a cotton ball and adhesive tape. About 5–6 drops of 50% corn syrup solution is placed on top of the nylon screen and the jars along with a wet sponge are placed in plastic boxes with a tight fitting lid. The boxes are placed inside the incubators for about a week to allow females enough time to oviposit. The ovipositing females are given fresh 50% corn syrup solution (5–6 drops) daily. Within a week of transfer, most of the females lay their eggs on the plaster surface of the oviposition jars and die. The dead females are then sucked out using a glass-pipette attached to an air vacuum system.

(b) Immature stages

The number of eggs laid by each female varies according to species, rearing conditions, and the size and source of a blood meal. A female usually deposits anywhere between 5 and 50 eggs. After the dead females are sucked out of the oviposition jar, the jar is resaturated with water and the nylon screen cover is replaced with the plastic screw-on lid of the jar. Each jar is checked at this point for larval hatching, and a small amount of larval food (Section 3.2.2, step 5) is sprinkled onto the plaster surface using a wooden tongue depressor. Each jar is checked twice weekly thereafter for larval growth, food, moisture, mould and mites. Larval food is usually added to the jars during these observations unless most of the larvae are grown to 4th instar stage at which time they do not need any additional food. The larval (1st–4th instar) and pupal periods usually last for about 3–4 weeks and 1 week, respectively. When the adults start emerging in the oviposition/rearing jars, they are transferred to adult holding/feeding cages and the cycle is repeated.

NOTES

A number of factors described below are important in the care and maintenance of closed colonies of sandflies in the laboratory. Wherever possible, alternatives are included in the notes to facilitate procedures in developing countries where access to sophisticated equipment is limited.

1. The insectary is the base for rearing and maintaining sandflies under laboratory conditions. It can be a suitably sized room consisting of portable humidifiers, heaters and steel or wooden racks (instead of expensive incubators) for accommodating adult holding/feeding cages as well as plastic boxes containing oviposition/rearing jars.
2. The optimal temperature and humidity range for most species is between 23 and 28°C and 70 and 100%, respectively. The photoperiod can be (L:D) 12:12, 14:10, 16:8, 17:7. Some species can be reared in complete darkness.

3. The ovipositing/rearing jars may be made of earthenware pots, plastic pots (various sizes) or plastic/glass vials (for individual females) with a layer of plaster of Paris.
4. The adult cages can be of various sizes and made of nylon cloth bags supported by a plastic/steel frame.
5. Some sandfly species are very fastidious in their blood-feeding behaviour and may need to be fed on mouse, rat, rabbit, chicken, or man. Occasionally, a membrane feeder may have to be used if the females refuse to feed on any animal (Chapter 10; Maroli, 1985; Ward, *et al.*, 1978). The best practice is to adapt females for feeding on hamsters by regularly offering hamsters to hungry females for a few hours. This reduces the need for keeping several animals at a time in the laboratory for feeding females.
6. Instead of apples and corn syrup, a number of other sources of sugar (30–50% solution) can be used for feeding adult flies.
7. Larval food is a critical component of sandfly rearing. The discovery of ageing of larval food (Young *et al.*, 1981) was one of the major steps towards successful mass rearing of some species in the laboratory. The ageing process requires over a month, and each lot of larval food should be tested after it has been aged for fungal growth before use. This is done by placing a small quantity in a moist empty oviposition/rearing jar. If fungal growth occurs, the food should be saturated with water and aged for additional time.
8. The quantity of larval food and moisture is very critical during 1st and 2nd instar larval growth and should be controlled carefully. The amount of moisture is judged by looking at the plaster surface of the ovipositing/rearing jar under a dissecting microscope. If a thin shiny layer of water is seen on the plaster surface, it is just enough and excess should be blotted with a paper towel or cloth.
9. Laboratory colonies are known to be infested by mites, ascogregarines, nematodes, viruses and fungi. Care should be taken to avoid cross-contamination with these pathogens. Maintaining cleanliness in laboratory cages, tools, and equipment helps in avoiding infestation.
10. Several other factors such as extreme temperature and humidity, poor larval diet, overcrowding of the larvae, diapause in 4th larval instar, females refusing to feed, eggs retained by gravid females, death of 1st–4th instar larvae, death of pupae, death of engorged females, laying of infertile eggs, etc., may interfere with the productivity of a colony and should be carefully monitored.

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CHAPTER FOUR

Care and maintenance of blackfly colonies

Eddie W. Cupp and Frank B. Ramberg

4.1 INTRODUCTION

The availability of vector species on a continuous basis in the laboratory is an important prerequisite for both basic and applied aspects of research. Unlike other types of filariasis vectors (particularly mosquitoes), the immature stages of the Simuliidae (blackflies) are found exclusively in riverine or other types of running-water habitats. In the laboratory, duplicating this unusual aspect of the biology of the immatures (especially the larvae) poses a major obstacle to colonization of most important vector species. Thus, sustained propagation of many medically-important taxa under artificial conditions is difficult, if not impossible, at the moment. While several species of Simuliidae have been reared successfully through multiple generations in the laboratory, there are currently no colonies of a natural vector.

This lack of vector material in many cases has necessitated the use of nonvector 'model' species as replacements, or required the 'partial' rearing and/or maintenance of field-collected vector insects in the laboratory for experimental purposes. In the latter situation, knowledge gained by working with model species has been useful and, in some cases, critical to the success of the investigation where vectors were involved. Alternatively, the adults of vector species can be collected in endemic onchocerciasis foci and transported relatively long distances for further investigation. While sometimes costly, this approach has been used quite successfully to compare the vector competency of both African and Latin American *Simulium* species for *Onchocerca volvulus* and determine biochemical and molecular aspects of salivary gland activity (Cupp *et al.*, 1995).

4.2 MATERIALS

Rearing/colonizing simuliid species under ordinary laboratory circumstances differs fundamentally from other vector arthropods in that a reliable closed system of water recirculation is required to promote development of the immature stages. Because of this peculiarity and the lack of commercially available equipment for this task, dif-

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ferent types of specialized rearing devices and ancillary equipment have been handmade and used successfully over the past several decades for this purpose. Furthermore, blackfly adults require special handling techniques in the laboratory to induce mating, blood-feeding and oviposition that are often quite unlike the methods used for other more common vectors such as mosquitoes.

4.2.1 Rearing systems

Brief descriptions of general rearing systems have been reviewed in several publications (Edman and Simmons, 1985, 1987). Listed below are the materials and equipment used for three types of rearing systems that differ widely in their complexity and production capabilities; each system can be used in the laboratory to produce material for experimentation.

1. Magnetic stirrer system (Grunewald, 1973; Colbo and Thompson, 1978): 1 L (or greater) glass beakers for larvae to attach to sides, and a magnetic stir bar to create a vortex current.
2. Compressed air rearing system (Simmons and Edman, 1981; Lacoursière and Boisvert, 1987): A hand-constructed, plexiglass apparatus with internal sliding plates for larval attachment and a piston-driven standard aquarium pump to provide aeration.
3. Trough system (Raybould and Grunewald, 1975; Brenner and Cupp, 1980; Brenner *et al.*, 1980; Bernardo *et al.*, 1986): The type of container that serves as the central reservoir can vary (garbage cans, glass aquaria, 643 L Dayno Aqua-Lab with water temperature control). The trough can be handmade of plastic or wood which allow larvae to attach. One system (Brenner and Cupp, 1980; Brenner *et al.*, 1980; Bernardo *et al.*, 1986) has an automatic feeding device that uses a glass aquarium as a food reservoir and two submersible pumps controlled by Tork timers to deliver food into the trough.

4.2.2 Larval food

Three diets are listed below, all of which are acceptable. They may be prepared by milling or grinding and are generally used as the main diet with bacteria and algae that occur naturally in the rearing system.

1. Tetra fish food (Colbo and Thompson, 1978).
2. Low-fat rabbit food (Brenner and Cupp, 1980).
3. Low-fat rabbit food/soybean protein (Bernardo *et al.*, 1986).

4.2.3 Collection of adults to promote mating and blood-feeding

1. Glass funnel.
2. Tygon tubing.
3. Aspirator.
4. Small plastic tubes: approximately 100 mm length by 25 mm diameter.
5. Membrane material: animal skins, rubber, artificial membranes (i.e. Parafilm).

6. Feeding chambers: water-jacketed glass membrane feeders (Rutledge *et al.*, 1964) can be custom-made (D. Lillie, Inc., Smyrna, GA) to vary in fluid capacity and size of membrane to be exposed.

4.3 METHODS

For a variety of reasons, blackflies can be very difficult to work with in the laboratory; thus some preliminary experimentation is required prior to engaging in a major effort to colonize a species. It is therefore important to first gain experience in rearing the immature stages, maintaining the adults, inducing blood-feeding by adult females, and implementing methods that consistently induce oviposition of fertilized eggs.

Because of the long-term success in maintaining *Simulium vittatum* in the laboratory (currently in its 14th year of continuous colonization and in the 120th generation), the knowledge and experience gained from rearing that species serves as an excellent guide for general methods and techniques that can be adapted and used for other simuliids. In addition to the methodological information provided here, the

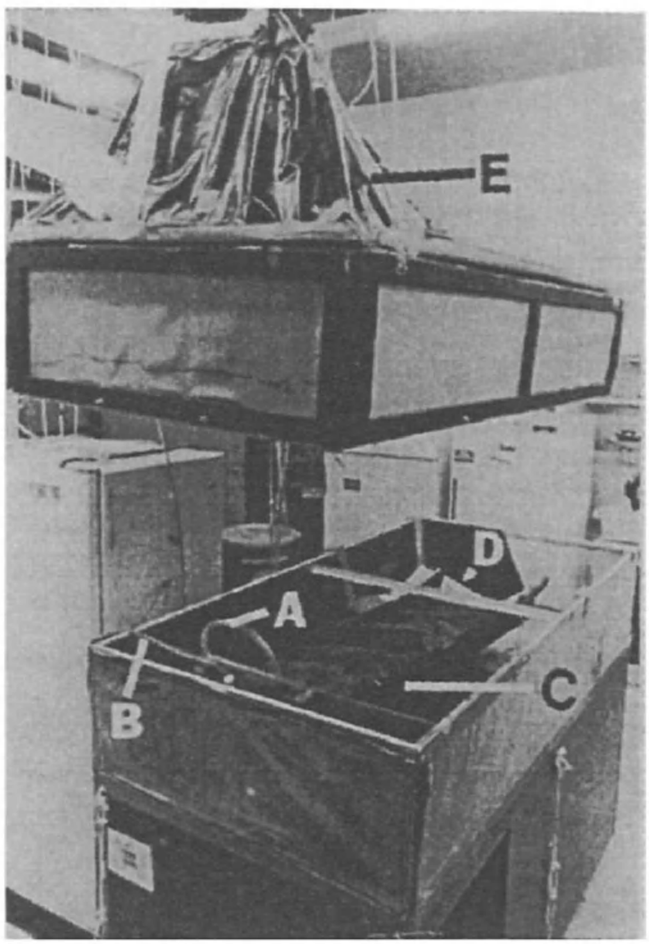


Figure 4.1 Photograph of Brenner and Cupp (1980) trough rearing system. (A) Antisiphon tube for automatic feeding system; (B) food tube (from refrigerated food reservoir); (C) trough (raceway) with baffles; (D) filter system; (E) opaque tower (the emergence trap on top of the tower is not shown). (From Edman and Simmons, 1985.)

reader is referred to publications by Bernardo *et al.* (1986) and Bernardo and Cupp (1986) for other details. The method described here is based on the use of a modified Dayno AquaLab trough system described above in Section 4.2.1, step 3 and pictured in Figure 4.1.

4.3.1 Rearing of immature stages

Listed below is the general method for rearing *Simulium vittatum*; (Notes 1 and 2 for more information about the necessary size of the rearing apparatus relative to the number of individuals).

1. Day 0:
   a. Water temperature: ambient (20–22°C).
   b. Water velocity (in trough): trickle.
   c. Food: none.
   d. Oviposition cloths containing embryonated eggs are 'pinned' to the top of the trough.
2. Day 1–3:
   a. Water temperature: ambient (20–22°C).
   b. Water velocity (in trough): trickle.
   c. Food: 100 mL of powdered food (50% soy meal, 50% rabbit food) filtered through a 53 μm metal sieve; 7–14 L (2 g/L of suspended particles) added by hand each day to the reservoir.
3. Day 4–7:
   a. Water temperature: ambient (20–22°C).
   b. Velocity: increase to 0.25 of maximum output.
   c. Food: begin automatic feeding system; 6–7 L/day (250–300 mL/h).
4. Day 8:
   a. Water temperature: decrease temperature 3–4°C/day until it reaches 14–15°C.
   b. Velocity: increase gradually each day until maximum is reached.
   c. Food: for 4000–7000 larvae, use feeding regimen as described for day 4–7; for rearings ≥7000 larvae, increase feeding regimen to 15 L/day (625 mL/h) (Note 3 for details regarding larval nutrition).
   d. The first evidence of pupation will occur around day 15–17 (Note 4 for details regarding the pupal stage).

This general procedure, with slight modifications, has been used to successfully rear the immature stages of a number of North American and African species to produce healthy, vigorous adults. Once adults are produced on a consistent basis, it is also necessary to address a perennial problem – the question of inducing mating behaviour that leads to a high rate of insemination of the laboratory-reared female flies. Because of this difficulty, it may be necessary to use field-collected stages (larvae, adults) of vector species for experimental purposes instead of trying full-scale colonization.

4.3.2 Collection and maintenance of adults

1. In order to collect emerging adult flies, the rearing system is covered by a light-tight, cloth hood that usually consists of a black canopy placed over the rearing apparatus. Because adults are attracted to light, a collection device made up of an inverted glass funnel into which clear tygon tubing has been inserted is

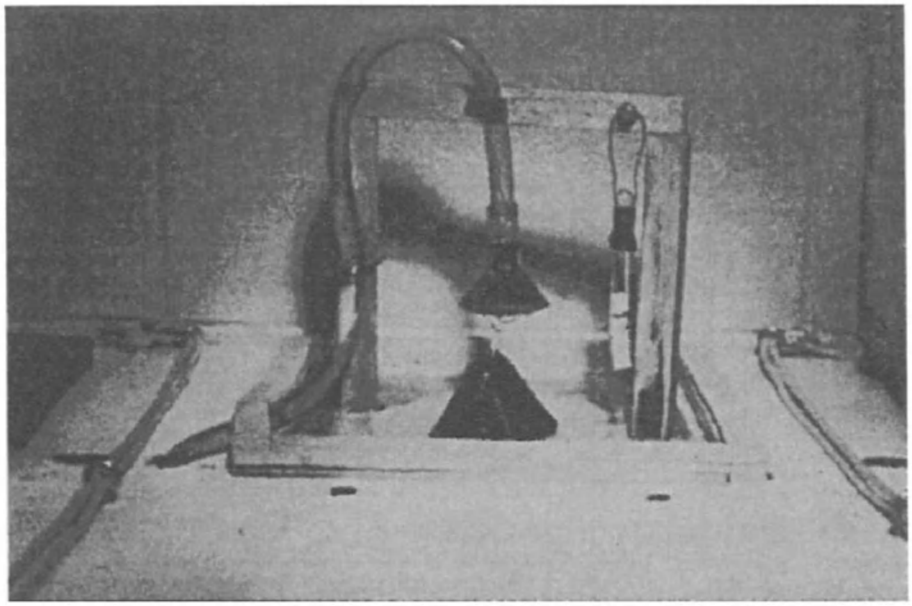


Figure 4.2 Laboratory oviposition environment developed for *Simulium pictipes*. Eggs are deposited on the black cloth draped over the inverted funnel. (From Bernardo *et al.*, 1986.)

placed on the top of the canopy. Adults (both sexes) emerge and fly/crawl into the tubing during daylight hours (Brenner and Cupp, 1980; Brenner *et al.*, 1980). Alternatively, adults can be aspirated directly from the sides and top of the rearing device.

2. The emergence hood should be lowered over the rearing unit 2 days after the first pupae are observed. Adults (males) will emerge between days 17 and 21. Feeding should be terminated when most larvae have pupated.
3. Since adult males appear about 24–36 h before female emergence begins, they should be removed and temporarily stored in the refrigerator until the females emerge.
4. The adult males and females are collected by an aspirator and placed together in a plastic tube to promote contact (Wenk, 1965; Wenk and Raybould, 1972).
5. A ratio of at least 2:1 (males to females) should be maintained in order to stimulate mating activity and provide sufficient opportunity for insemination to occur.
6. The adults are confined in a specific area of a darkened cage using a light source to stimulate congregation and promote contact.
7. Newly-mated females can be identified by the presence of a spermatophore covering the genital pore.
8. Once mating has occurred, a stream of humidified CO_2 can be used to anaesthetize the adults. Males can be placed in pint ice cream cartons in a humidified chamber and stored in the refrigerator for additional mating attempts. Inseminated females are then available for blood-feeding.

4.3.3 Blood-feeding and oviposition

Because of difficulties in using intact animals as blood sources for blackflies, an *in vitro* system has been developed which is effective for a number of blackfly species (Bernardo and Cupp, 1986; Note 5).

1. Containers holding inseminated females are situated so that the flies can access the artificial membrane of the membrane feeder (Notes 6 and 7).
2. The flies are allowed to feed until replete on a protein food source such as blood, serum, or serum and cell culture medium.
3. Blood-fed females are transferred into standard steel mesh cages used for holding insects (Note 8 for alternatives).
4. The cage is modified by introducing a source of running water through the top. The water falls to the bottom of the cage and splashes over and through an oviposition surface (Figure 4.2). This surface may be made from various objects (piece of tygon tubing, inverted funnel, etc.) wrapped with a rectangular piece of coloured polyester cloth (green, black, etc., depending upon the preference of the species – Note 9). The cloth provides a moistened substrate for oviposition of fertilized eggs. Various factors have been shown to have a significant effect on oviposition behaviour (Notes 10–13).
5. Alternatively, a floating cork or other type of dampened substrate may be used to induce egg-laying (Note 14).
6. Once oviposition has occurred, the polyester cloth can be removed, placed in a moistened petri dish, and held for 2–3 days until embryonation is completed.

4.3.4 Storage of blackflies

1. Eggs may be stored on wet ice at 2–3°C for several months in moistened petri dishes sealed with Parafilm (Note 15).
2. Adults (both sexes) of holarctic species can be stored in refrigerators at 2–3°C if suitable moisture and access to an energy source (carbohydrate) is available; males maintain their vigour for 2–3 weeks and can be used to augment numbers to improve mating/insemination rates for establishment of colonies (Note 16).

4.3.5 Field collection and shipment of blackfly species

1. In some instances, it is easier and more cost-efficient to collect blackflies from their natural habitats and transfer them to the laboratory for experimentation, than to invest in colonization. Thus a strategy of 'partial rearing' of field-collected eggs, larvae or pupae can be followed using the materials, equipment and methods described above to produce adult flies.
2. Eggs can be kept viable for reasonable periods of time (3–4 days) in transit and while embryonation is occurring by keeping them moist and slightly chilled. This approach also works quite well for pupae. Larvae are more difficult because they require aeration, thus limiting the amount of time that they can be in transit.
3. An alternative approach is simply to collect the adult females of a vector species and return them to the laboratory. To do so, daytime collections are made of flies attracted to a human host. Where information is available, collections can be conducted during the time of day in which the predominant proportion of the vector population is nulliparous, hence reducing the chance of parasite transmission to the collectors. Flies can be captured individually as they land on the host, tentatively identified, and then packaged for transportation.

4. The method described by Figueroa-M *et al.* (1977) should be used to prepare plastic tubes for collection and to package material for transit. Briefly, a rectangular piece of filter paper is inserted into small, clear plastic serum vials to create a resting platform for the adult fly along the length of the tube. Depending on the blood-feeding habits of the vector of interest, several hundred flies can usually be collected within a few hours.
5. Once the flies have been collected into the tubes (one fly per tube), a piece of plastic mesh is placed over the end of each tube and on top of this is placed a small piece of cotton soaked in sterile 15% dextrose + 1% penicillin–streptomycin–mycostatin.
6. The cotton should be glistening with moisture but not dripping the solution. The cap is then snugly snapped onto the tube to hold the cotton in place. This provides both a source of carbohydrate to the fly *en route* and a localized moisture source to prevent desiccation.
7. The tubes are then placed into plastic ziplock bags (15–20/bag), wrapped with moistened cotton wash cloths (glistening but not dripping wet), and then placed into plastic Igloo carriers for the return trip.
8. A thin paper layer (newspapers are excellent) is placed over this material and two frozen 'blue ice' packs are placed on top of the paper. This creates an environment during transit of ~ 21°C with ~ 75% RH. Survival rates for African and Latin American vector species transported over a 24 h period varies between 70 and 80%.

NOTES

1. The type and size of the rearing apparatus to be used depends on the number of larvae to be reared and the general purpose of the laboratory investigation. For example, rearing small numbers of larvae from field collections (or eggs) to the pupal or adult stage can be accomplished by using a simple plastic reservoir made of a plastic rat cage with an aquatic submersible pump (Tarrant *et al.*, 1988). Water is then recirculated over a small trough that is inclined at a slight angle; food (rabbit food/soybean meal described above) is added by hand at defined intervals. Emerging adults are collected by placing a black canopy over the rearing apparatus as noted above.
2. The use of larger rearing systems for consistent mass production of larvae and adults requires a much greater investment of financial resources, laboratory space and effort. However, with careful planning, colonies of selected blackfly species can be established. Colonies of *S. vittatum* are currently being maintained at the University of Arizona and Clemson University.
3. Some comment on the nutritional requirements of the larvae is instructive. Indeed, as is the case for rearing other filariasis vectors, adequate larval nutrition of the vector is the key to successful colonization. In this regard, a series of experiments demonstrated that blackfly larvae develop into highly fecund and vigorous adults when fed a diet of low-fat rabbit food supplemented by soy bean protein in a mixture of 1:1. Survival and adult size were maximal when larvae received a mean of 4 mg of food/larva/day. Prior to introduction into the rearing system, the food was washed through a series of sieves so that the average par-

ticle size (mass median diameter) of the mixture was small enough to be easily trapped by the filtering mouthparts. This is an important biological aspect when considering colonization of a species.

4. The simuliid pupal stage is encased in a silken cocoon that anchors the insect in a column of running water. This stage continues to feed at the onset of pupation but then becomes quiescent. If removed from the aquatic environment *via* field collection and kept on moist filter paper in Petri dishes, development can be completed. At usual laboratory temperatures (16–17°C), the pupal stage lasts about 3–4 days.
5. The physical aspects of the system described by Bernardo and Cupp (1986) are similar to those described here. The flies are fed inside a darkened incubator set at 16–17°C. As a result of these differences, a temperature differential is created between the surface of the membrane (which contains blood or tissue culture medium + bovine serum heated to 37°C) and the ambient temperature in the incubator. The flies are attracted to the heated surface, congregate on the membrane and then probe into it. Tasting and engorgement then follow.
6. Adult females will puncture and feed through a variety of membrane substrates including animal skins, rubber, and artificial membranes (Parafilm). General references on blood-feeding include Sutcliffe and McIver (1975), Bernardo and Cupp (1986) and Wirtz and Raybould (1986).
7. Engorgement rates can be significantly improved by adding ATP and lowering the ambient temperature to attract females to the warm membrane surface.
8. An excellent technique for maintaining adult females, particularly following their engorgement on blood or some other protein source, involves placement of flies into individual cells constructed by cutting holes into styrofoam sheets (Raybould and Mhiddin, 1974). The ends of the cells are covered with a fine mesh and the styrofoam blocks are then placed into garbage cans that contain a styrofoam substrate floated on about three inches of water (Bernardo *et al.*, 1986). This method allows the free movement of humidified air over the flies but avoids the problem of collection of drops of moisture to trap the insects. Also, when in darkness, most simuliids are inactive and therefore do not damage themselves by excessive flying and crashing into the walls of the cage.
9. The cloth may cover an object (piece of tygon tubing, inverted funnel) to provide a distinctive shape; gravid females deposit eggs directly onto the coloured cloth. The colour of the substrate is of some importance in initially attracting the females to the moistened surface. Thus, field observations on the colour and texture of oviposition substrates used preferentially by the species to be reared/colonized are important. Additional pertinent references include Brenner *et al.* (1980) and Simmons and Edman (1981).
10. Oviposition behaviour is enhanced in species that lay egg masses by an aggregation/oviposition pheromone (McCall, 1995). Decreasing light intensity is also very important in stimulating oviposition activity for many species.
11. The time of day in which oviposition normally takes place in nature can be critical in inducing oviposition in the laboratory. In the case of *S. vittatum*, flies are put into the oviposition chamber at dusk, which corresponds to the time in which gravid females initiate egg deposition.
12. There is also some variability in the behaviour of certain medically-important species that makes inducing oviposition in the laboratory difficult. For example, females of *S. ochraceum* (an important vector species in Mexico and Guatemala)

deposit eggs singly while hovering above the water's surface. This type of behaviour makes it difficult to obtain fertilized eggs on a scale large enough to sustain the species in the laboratory.

13. For most species, the eggs accumulate in masses and are held together by a gelatinous matrix. This type of ovipositional behaviour is exhibited by many species, including members of the *Simulium damnosum* species complex.
14. Attempts to induce oviposition by wetting gravid females and shaking them in a vial of water have not proven reliable. This problem exemplifies the types of elementary difficulties one often encounters with this group of insects.
15. While eggs can be held in the refrigerator (2–3°C) or on wet ice for up to 2 months before significant mortality occurs, as is the case with certain mosquitoes (*Anopheles* and *Culex* species for example), simuliid eggs will hatch almost immediately after the embryo is fully formed.
16. Additional information pertinent to storage can be found in Bernardo *et al.* (1986) and Edman and Simmons (1987).

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CHAPTER FIVE

Care and maintenance of tsetse colonies

Ron H. Gooding, Udo Feldmann and Alan S. Robinson

5.1 INTRODUCTION

5.1.1 Classification, distribution, and economic importance

The genus *Glossina* contains 31 taxa (21 species and eight subspecies), assigned to four subgenera (*Glossina, Nemorhina, Austenina, Machadomyia,* i.e. the *morsitans, palpalis, fusca* and *austeni* groups respectively). Several of the subspecies can be hybridized and this results in varying degrees of sterility. The genus is restricted to subSaharan Africa where its distribution can be divided into three zoogeographical areas, West, Central and East Africa. Tsetse colonize many different habitats within these areas, ranging from savannah to primary forest. Because tsetse are the vectors of protozoan parasites belonging to the genus *Trypanosoma* in Africa, they have a major impact on humans and domestic animals. Devastating epidemics of the human form of the disease, sleeping sickness, have frequently been recorded and currently over 10 000 new cases are diagnosed annually. However, the animal form of the disease, nagana, has been pivotal in preventing the agricultural development of large tracts of Africa and it is estimated that the presence of tsetse limits the use of 7 million km^2 of grazing land.

5.1.2 Life history of tsetse: constraints upon colony maintenance

Female tsetse are viviparous and each female produces a fully grown larva every 9–10 days. As this larva weighs more than the 'mother' at birth, it is clear that the female must have access to, and be able to metabolize, a large nutrient supply. This she finds in the form of a regular supply of vertebrate blood. In contrast to many other insect disease vectors, the males also feed exclusively on blood. The provision of high quality, non-contaminated blood is the major requirement for successful tsetse rearing in the laboratory.

Ovulation proceeds alternately from two polytrophic ovarioles and the egg is fertilized by sperm from the paired spermathecae. The larva is nourished by secretions from the uterine gland which contain initially acidic lipids, but later the balance

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changes to include more protein. There appears to be no connection between the larval midgut and hindgut thus preventing the loss of nutrients through the anus. The time taken from ovulation to larviposition is about 9 days at 25°C, comprising 4 days for the egg stage and 1, 1.5 and 2.5 days for the 1st, 2nd and 3rd larval instars, respectively. At completion of the 3rd instar, larviposition takes place, followed within 2 h by pupariation.

The puparial stage is long, taking up to 34 days; for males the puparial stage lasts 1–2 days longer than it does in females. Adults emerge from the puparium by using the ptilinum to force the cap off the puparium. Unfed flies are referred to as teneral and during this phase they are relatively soft.

Males become sexually mature about 1 week after eclosion, whereas females of most species take only 2 days. This difference in sexual maturation time can have important consequences for colony maintenance as males and females are generally placed together for mating only after attaining sexual maturity. This is done to ensure that females do not mate with immature, aspermic males and thus become refractory to subsequent mating attempts by mature males. The correct copulatory response is mediated through a contact pheromone which is present in the cuticular wax of the female. Copulation can take several hours during which time the male transfers all the components of the spermatophore followed by the spermatozoa.

5.1.3 Uses of tsetse colonies

The agricultural and medical importance of tsetse has been the driving force to develop efficient laboratory rearing procedures for these insects. Large numbers of high quality flies are needed to develop new control technologies and to study the interaction between the trypanosome and the fly. The unusual reproductive biology of the tsetse has also been of interest to insect physiologists.

The development of vector control strategies always includes a significant laboratory component during which candidate compounds are screened for their particular effectiveness; these can be attractants, insecticides or juvenile hormone analogues. Bioassays using these compounds must be carried out using flies of standard physiological age. Considering the large changes that females undergo during the pregnancy cycle, it is essential to have a well maintained colony in order to carry out meaningful experiments.

During the last 20 years tsetse colonies have been developed to provide males for the Sterile Insect Technique (SIT). In this control approach, large numbers of flies are reared, and the males are sterilized and released. The low reproductive rate of tsetse in the field makes them ideal targets for this technique, as has been successfully demonstrated in West Africa (Cuisance *et al.*, 1986; Oladunmade *et al.*, 1990). Of course the low reproductive rate, that is a benefit for application of SIT, is a handicap when it comes to rearing tsetse. Nevertheless, *G. austeni* colonies of up to 250 000 reproducing females are now supplying flies for the eradication of this species on the Island of Zanzibar. Significantly, one of these colonies is in Tanga on the Tanzanian mainland.

At present the technology used to maintain large colonies differs very little from that used to maintain research colonies. Significant expansion of large field programmes will require an extensive revision of the mass-rearing process.

5.1.4 Strategies for establishing tsetse colonies

Tsetse colonies can be initiated with field-collected material or material from an existing colony. These are quite different strategies, each having advantages and disadvantages. Whichever procedure is followed, importing and maintaining tsetse colonies requires special permits in some countries, and we recommend that the appropriate regulatory agencies be consulted before any effort is made to establish a colony.

Tsetse colonies can be established with field-collected puparia, or by collecting females and maintaining them long enough to produce puparia. The latter procedure carries the risks of maintaining flies infected with vertebrate pathogens. Some field-collected puparia may contain dipteran or hymenopteran parasitoids, but there is very little risk of these becoming accidentally established in a laboratory. Puparial collections ensure that no vertebrate parasites are included in the sample. In general, obtaining sufficient field-collected material to ensure an adequate sampling of the gene pool is problematical for most species. Once the flies are in the laboratory, any difficulties encountered in feeding them or getting them to mate will very quickly lead to loss of the colony. Making large field collections can be time consuming and expensive. Therefore it is recommended that every attempt be made, prior to the arrival of the puparia or flies, to assure that the required rearing conditions are available.

Obtaining a starter colony from an established colony is clearly the easiest procedure to follow. The flies are laboratory adapted, their productivity is known and additional puparia are readily available in case of a failure. However, there are potentially several unknowns in this procedure, the most important of which are the identity, history, and integrity of the material that is received. By identity we mean the data necessary to pinpoint the original field source of the material. Geographical variation in many important physiological processes indicate the importance of these data. The post-colonization history of strains sometimes gets lost, making it impossible to track the path of a particular strain through a series of laboratories. It is also important to know if the strain has been supplemented with field-collected material at any time and, if so, from where the field material originated. The preceding indicates the importance of good documentation during colony establishment and maintenance.

5.2 MATERIALS

Manipulation of tsetse *per se* is composed of collecting puparia, collecting and sexing teneral adults, placing flies together for mating and subsequently separating the sexes (if deemed necessary). Feeding is done by placing cages of adults on a source of blood. Compared to other insects, tsetse colonies require a relatively large amount of space. This is influenced by the type of cages used to hold the adults, and the method of feeding the flies. To minimize the amount of space needed, it is important to consider carefully the style and size of cages (for holding adults), trays (for holding cages), and feeding surfaces, so that these are compatible with each other. Furthermore it is important to choose a system that facilitates transfer of flies between cages. Finally the choice of cages, etc. will be influenced by the intended size and use of the colony.

5.2.1 General information on materials

Most materials can be obtained or made locally and we list only those items to which special attention must be paid.

1. Terylene netting (Material No. 6480, 48″ black): John C. Small and Tidmas Ltd., Perry Road, Sherwood Nottingham, England NG5 3AF.
2. Acrylic netting (white, Article #3029/2.5 mm square holes): E. Fellinger u Co., A-1013 Wien, Neutorgasse 5, Austria.
3. Silicone (Wacker RTV 539; Harter T35 rot): Pointner and Rothschadl, A-1040 Wien, Theresiamungasse 7, Austria.
4. Heating mat (150 × 50 cm): Murbauer, A-1150 Wien, Goldschlagstrasse 52, Austria.
5. Rabbit for *in vivo* feeding: A cross of Flemish giant and French lop-eared rabbits is probably the most useful laboratory host. Docile animals with relatively large floppy ears are the best hosts.
6. Blood for *in vitro* feeding: Defibrinated bovine blood is used to feed *G. austeni* and *G. pallidipes*; for other species use a mixture of 75% bovine blood and 25% pig blood (Notes 1 and 2 regarding collecting blood and checking blood quality).
7. Plastic Petri dishes or plastic 35 mm film canisters: for shipping/transporting fly pupae.

5.2.2 Tsetse cages

All cages must be covered with a cloth mesh through which flies may feed and through which larvae, but not adults, may pass.

1. Netting: For most species, a suitable material is 250 denier black terylene netting (i.e. 16 holes/cm^2); for large species such as *G. brevipalpis* acrylic netting with 2.5 × 2.5 mm holes must be used. The netting may be sewn into sleeves that are just large enough to permit insertion of metal frame cages, or the netting may be glued (using PVC cement) to frames made from PVC tubing or plastic vials. To glue netting to cages, the netting is held taut with an elastic band before applying the glue. Whichever cage style is used, care must be taken to ensure that the holes in the terylene netting are more or less circular and not strongly elliptical, as larvae may not be able to pass through the latter.
2. Small cages: Cages for individual flies may be of metal or plastic. Metal cages are constructed from 3 × 3 cm aluminum conduits (with 2.5 mm thick walls) that are cut into 5.5 cm lengths. The bulk of each surface is removed by milling and the metal frame is inserted into a 4.5 cm wide × 20 cm long terylene netting sleeve that is sewn along one side and one end. Plastic cages are made from transparent vials (3.8 cm diameter, 6.0 cm long, with a 1.5 cm high cap that fits into the vial). The vials are modified by replacing the bottom and centre portion of the cap with netting.
3. Large cages: Cages for 15–20 females or 20–40 males may be made of metal or PVC tubing. The metal cages are constructed from four stainless steel rods (3 mm diameter, 15 cm long), that are welded (using general purpose silver brazing rod [Eutecsil 1030 FC]) to the corners of two stainless steel plates (16 gauge, 5 × 7.5 cm). For maximum strength a 1 cm wide flange is folded back at a right angle toward the surface of the plate onto which the rods are welded. The terylene netting sleeve is taped to the cage using adhesive tape that is water resistant. Circular PVC cages (to hold 20 females or 40 males) are made from PVC

tubing that is 3–4 mm thick and 11.5 cm diameter and cut into 4.5 cm lengths. Oblong PVC cages are made from the same type of PVC tubing that is heated to 60°C in water and reshaped to ca. 13.5 cm long × 6.5 cm wide by placing a weight on the tubing. Circular cages for 100 females are made from 20 cm diameter PVC tubing (4 mm thick) that is cut into 5 cm lengths. All cages, except those for holding individual flies, have a 2.5 cm diameter access hole in one end of the cage. Access holes are closed by cork or rubber stoppers. Metal cages and the oblong PVC cages are suitable when flies are fed on rabbits, but, in order to minimize disturbing the rabbits, each cage should hold no more than 17 flies. All cages may be used when flies are fed through artificial membranes.

4. Emergence cages: Metal-frame or oblong PVC cages, fitted over the open ends of glass tubes containing puparia may be used as emergence cages. For handling large numbers of flies, emergence cages are constructed from 10 cm long pieces of 20 cm diameter PVC tubing that have been reshaped (in water at 60°C) to form rectangular cages approximately 13 cm high and 22.5 cm long. The height of the cages must permit air to be blown into the cages when they are placed in the chiller (Section 2.4). A 6 cm diameter hole is cut in the bottom of the cage to accommodate the Petri dish holding 400–500 puparia. Two pieces of plastic are attached to two sides of the hole to provide a groove along which a piece of plastic is inserted to close the hole.

5.2.3 Trays for holding cages

1. Trays may be made from metal, plastic, plexiglass, or any other readily cleaned material, and should be constructed in such a way that only a small portion of the bottom of each cage is in contact with the tray. This may be accomplished by having two appropriately placed ridges or rods (on which the cages rest) as an integral part of each tray or by having a metal grid above the tray. Such an arrangement is desirable for all cages and must exist for cages holding breeding females to permit larvae to escape from the cage and reach a suitable site for pupariation.
2. When numbers of flies warrant it, breeding females should be held on metal grids over slanting trays that have a trough at their lower edge. This permits larvae to reach the trough where pupariation occurs. To facilitate collection of puparia, the trough may be detachable or it may have a hole at the one end through which puparia may be swept with a small paint brush. The hole is closed with a cork or rubber stopper when not in use. For small colonies (i.e. up to 3000 flies), females may be held in plexiglass trays that have two ridges (2 cm high) to support the cages. Collection of puparia is made easier by placing a 1-cm-deep layer of medium or coarse grain sand in the tray.
3. In large colonies, and whenever an *in vitro* feeding system is used, the trays holding cages of flies should be mounted on trolleys that can be moved from the holding facilities to the feeding room.

5.3 METHODS

5.3.1 Environmental requirements for holding flies

1. Adults of most species should be maintained at 24°C, and fairly humid conditions (75% RH for subspecies of the *morsitans* group; ca. 80% RH for *G. tachinoides*

and *G. brevipalpis*; and 85–88% RH for *G.f. fuscipes, G.p. palpalis* and *G.p. gambiensis*).

2. Since flies are disturbed by nearby movements, adults should be maintained under subdued, indirect lighting, preferably using tungsten lamps on a 12:12 photoperiod. However, colonies may be maintained under fluorescent lighting in rooms in which human activity is minimal.
3. While cages are in the holding facility, the surface through which feeding occurs must never be at the bottom of the cage.
4. Puparia should be maintained under the same conditions as adults, except puparia of *G. tachinoides* which do better at 23°C and 82% RH, and puparia of *G. austeni* which do better at 85-88% RH after they are 20 days old.
5. The conditions in the feeding environment are the same as those used for maintaining adults. However, for large colonies, and when an *in vitro* feeding system is used, it is desirable to have separate holding and feeding facilities in separate, but adjacent, rooms.
6. For *in vitro* feeding, the feeding room must be kept under aseptic conditions (e.g. with UV light over the feeding areas) overnight prior to feeding, or the area must be periodically cleaned with an antiseptic solution.

5.3.2 Environmental requirements for manipulating flies

1. Adults may be manipulated without immobilization by taking advantage of their tendency to move toward light. By covering their cage with a dark cloth they may be enticed to move into a glass tube that just fits into the access hole in the cage. With practice they may be moved from one glass tube to another, if the worker can effectively close the tube opening with his/her thumb.
2. Individual flies may be removed from rectangular metal-frame cages by partially blocking the access hole with cotton wool and inserting a rigid-walled plastic tube (the type used to store laboratory thermometers) into the cage and patiently coaxing the fly to the far end of the cage before trapping the fly in the tube.
3. For the PVC cages, it is necessary to extract individual flies by using a thick-walled glass tube that has an outside diameter slightly smaller than the access hole in the cage and that is slightly bent at one end and covered with gauze or netting at the other end.
4. The above procedures are very time-consuming, and for routine manipulations flies are immobilized by exposure to cold (2–4°C) or nitrogen. The latter is slower and more expensive than the former.
5. When cold immobilization is used, the flies must not be immobilized until several hours after feeding, and at least 1 hour before feeding.
6. For best results, flies are not fed on the day when they are chilled. In all cases do not chill flies for longer than is necessary to carry out the manipulations needed. This is particularly important if flies are immobilized in a stream of cold air.
7. For small colonies, flies may be immobilized by placing cages on a tray within a standard, commercially available machine for making crushed ice. The flies are then transferred to glass tubes which are held briefly in crushed ice.
8. Teneral flies and those that have not been fed for at least 24 h may be cooled to near 0°C for up to 20 min.
9. For manipulating large numbers of flies, a space for their cold immobilization may be constructed by modifying a –20°C deep freezer as follows. The unit must

be equipped with a heavy duty compressor that is designed to operate at 0–6°C. The lid is removed and the temperature controls are replaced with a thermostat to produce an air temperature of 2–4°C in the working area described below. A turbo-style electric fan, capable of generating a near-laminar flow, is installed on the floor at one end of the freezer. The fan should be equipped with a rheostat to adjust airflow so that enough air is delivered to immobilize flies without blowing them about on the working surface. The freezer is fitted with a stainless steel tray that is suspended from the walls of the freezer or supported from its floor. For 15 cm at each end of the deep freezer the top of the tray is level with the top of the freezer. Between these areas the floor of the tray is 20 cm lower. At the end above the fan, the tray is equipped with adjustable louvres that direct air down to a 20 cm wide work space on the floor of the tray. The work space is a plastic platform about 2 cm high, covered with a glass plate and equipped with at least two shoots (on the side away from the louvres) to facilitate transfer of flies from the work space to cages. At the end of the tray opposite the louvres, holes permit air to return to the freezer. Air flow within the freezer chest can be improved by installing a curved sheet of metal to direct air towards the louvres. The top of the work space is covered by a 40 cm high plexiglass hood. On one side of the hood, near the louvres, is a 20 cm high × 40 cm long access port that is covered with a piece of removable plexiglass. A cool-white fluorescent light, mounted in a metal holder, is placed on top of the hood to provide adequate lighting.

5.3.3 Feeding tsetse

(a) General comments on feeding flies

1. Flies should be fed under environmental conditions that are as close as possible to those under which they were held.
2. Lighting should be subdued, indirect and preferably from tungsten lamps. Human activity in the room should be minimal.
3. Cages may be covered with a lightweight, dark cloth to reduce disturbance of the flies, but care must be taken not to create a situation in which excessive heating of the cages occurs.
4. Both males and females must be fed blood at suitable intervals. The simplest schedule is to feed the flies every other day, including weekends and holidays. Other successful routines include feeding flies six consecutive days each week or feeding on Monday, Tuesday, and Thursday to Saturday.
5. Flies should be allowed at least 10 (and preferably 15) min to feed. Flies should not be fed on the day on which they are chilled, but colonies have been maintained in which they are fed one or more hours after being chilled.
6. Feeding young flies before the older ones minimizes the risks of bacterial infections spreading through colonies maintained by *in vitro* feeding.

(b) General comments on in vivo *feeding*

1. The methods originally developed to maintain tsetse colonies depended upon use of live hosts such as goats, guinea pigs, or rabbits.
2. If a reliable source of blood is available, we recommend use of the *in vitro* feeding system described below. However, when an assured supply of blood is not

available, or when the feeding procedures described below cannot be attained, it is possible to maintain tsetse colonies using an *in vivo* system.

3. The rabbits must be fed a high quality diet that is free of antibiotics and insecticides, and sick or injured animals that are being treated with antibiotics must not be used as hosts.
4. Rabbits that are about five months old are trained first to sit in restraining boxes (equipped with a soft pad), then to accept elastic belts across their backs, and finally to accept a yoke-collar and restraining (by large elastic bands) of their ears to padded, horizontal platforms that are fixed to the restraining box.
5. The two elastic belts that cross the rabbit's back are 2–3 cm wide and are stapled to one side of the box and fixed to the other by means of velcro pads.
6. All species of tsetse may be fed on the upper surface of the ears. All but the large species (*G. brevipalpis*, etc.) may be fed on the clipped backs of the rabbits, with the cages held in place by elastic belts.
7. Each rabbit may be used to feed about 400 flies/day, for up to about one week before being given a two-week rest. Rabbits will tolerate this regime for several years, but are terminated if they are unable to maintain their weight as they age.

(c) In vitro *feeding system*

The most efficient way to maintain tsetse colonies is by feeding flies upon sterile, freshly frozen, defibrinated or heparinized, blood through a silicone membrane (Bauer and Wetzel, 1976).

1. For membranes used to feed *G. austeni* or *G. tachinoides*, use terylene netting made of 130–150 μm diameter thread (62 holes/cm^2); for other species use netting made of 250–300 μm diameter thread (16 holes/cm^2).
2. The netting (60 × 60 cm) is cleaned by boiling in distilled water, air dried, and placed on a clean, smooth perspex plate (that is about the same size as, or slightly larger than, the netting).
3. For the thin membrane, thoroughly mix 150 g silicone and 7.5 mL of hardener (e.g. Wacker silicon TRV and hardener T35). (For the thick membrane use 20% more of each ingredient.)
4. Place the silicone/hardener mixture on the centre of the netting, cover it with a 70 × 70 cm transparent polyethylene sheet that is at least 0.3 mm thick and spread the silicone/hardener mixture evenly using a smooth, sturdy, plastic rod that is at least 70 cm long.
5. Allow the material to harden for 24 h at RT.
6. Remove the polyethylene sheet from the silicone membrane and then the membrane from the perspex sheet.
7. Cut off excess netting and the thick edges of the membrane.
8. Wash the membrane in hot, distilled water and either store it by rolling it up with paper towel, or use it immediately as indicated below. With proper care, membranes will last about 1 year.
9. Feeding trays are constructed of 1.2 mm thick anodized aluminium having either a 3 mm 'diamond'-shaped surface pattern, or are corrugated. The size of the tray will be determined by the cages used, heating apparatus, and ovens available for sterilizing the trays, but in any case the trays must have 1 cm high walls.
10. Heat blood to body temperature by placing the trays on a suitable heating apparatus that has a rheostat for controlling its temperature. Metal boxes containing

tungsten lamps, plexiglass or metal chambers through which warm water is circulated, or PVC heating mats may be used.

11. Feeding trays and the silicone membranes are thoroughly washed and rinsed with distilled water and the membranes are placed on the feeding trays.
12. The trays are stacked in an oven and left at 120°C overnight. (To avoid damaging the silicone membranes, care must be taken to ensure that each tray does not touch the membrane on the tray below it.)
13. Bottles of frozen, quality-screened blood (below) are thawed under cold running water, dried and taken to the feeding room. If blood is stored in 4–5 L polyethylene containers it will be necessary to allow the blood to thaw overnight.
14. Feeding trays, with their silicone membranes, are removed from the sterilizing oven, brought to the feeding room, and placed on the heating apparatus.
15. After turning off all air-circulating equipment in the feeding room, a corner of the silicone membrane is lifted to expose about two-thirds of the feeding tray and the blood is poured onto the middle of the tray (100 mL of blood is sufficient to cover 2000 to 2300 cm^2 and feed about 1500 flies).
16. The membrane is gently placed over the blood so as to avoid trapping air bubbles. Blood is distributed uniformly under the membrane by use of a plastic rod or a roll of paper towel.
17. A thermocouple or surface thermometer is used to confirm that the membrane temperature is approximately 35°C.
18. Cages of flies are placed with the feeding surface of the cage next to the membrane.
19. Immediately after use, the trays and membranes are thoroughly washed with cold tap water and rinsed with distilled water. Membranes are then placed in the trays and these are sterilized at 120°C overnight for use the next day.

5.3.4 Breeding tsetse

(a) Collecting and holding puparia

1. Puparia should be collected before they are 28 days old. The frequency of collection will be determined by the size of the colony; in large colonies they are normally collected each working day (Note 3).
2. Puparia are held, never more than four puparia deep, in tubes or Petri dishes. For at least the first 4 days, and preferably for the entire puparial period, the container must be open to the air (i.e. not stoppered or covered).
3. About 1 week before adults are expected to emerge, the container holding the puparia has its open end inserted into a cage into which the flies emerge. For small batches of puparia (i.e. up to 100 puparia), a 4 cm tall, 2.5 cm diameter tube is appropriate and emergence may be directly into the same type of cage that is used to hold adults. For large batches of puparia it is necessary to use an emergence cage, as described above (Note 4).
4. Puparial incubation conditions should be adjusted to a temperature such that the peak of female emergence takes place 32 days after larviposition.

(b) Sexing teneral flies

1. In order to obtain the maximum insemination rates in females, it is necessary to separate teneral males and females. Unfortunately there are no automated systems or genetic sexing systems to do this.

2. Sexing must be done manually on the basis of visual characters, and is most easily done when flies have been immobilized by chilling.
3. Males are readily distinguished from females on the basis of three characters that are visible to the unaided eye when the ventral surface of the abdomen is examined. In males the posterior end of the abdomen is pointed, the superior claspers or external genitalia are heavily sclerotized within a few hours of emergence, and the 'heart-shaped' fifth abdominal sternum is largely sclerotized and carries many setae. In contrast, the female's abdomen is truncated and its midline region is not sclerotized and has a pale appearance.

(c) Mating flies and holding breeding stock

1. Males do not reach full sexual capability until they are approximately 1 week old. Females of most species are maximally receptive to males when they are 2–3 days old; older females will mate but refractoriness increases with age.
2. Females of *G. pallidipes* are not sexually receptive until they are approximately 8 days old, and *G. brevipalpis* females are most receptive 1 day after eclosion.
3. Satisfactory mating occurs in most species by leaving slightly more males than females together for 1 or 2 days, and then chilling the flies and separating them.
4. For *G.p. palpalis* place young females in a cage with one-third their number of 1-week-old males and leave them together permanently.
5. If males are separated from females after mating, they may be used as studs two or three times.
6. Females are maintained for 13 to 15 weeks. Dead females should be removed from the cages at least once a week.
7. Tsetse are most efficiently maintained by expanding the colony to the desired size and then maintaining a stable age distribution of breeding females. This may be done by adding the same number of cages of mated females each week as are effectively removed at age 13–15 weeks.
8. If puparia are partitioned between 'colony maintenance' and 'other uses' then the number of puparia retained on a daily or weekly basis must equal twice the number of mated females that must be added to the colony during this period, plus enough puparia to account for mortality during the puparial stage and during the early adult life. In general between 45 and 65% of the puparia must be retained to sustain the colony and about 10% of the entire colony size needs replacement on a weekly basis (Notes 5–8).

(d) Transporting tsetse

1. If necessary, adults may be transported in standard cages that are adequately cushioned in insulated boxes.
2. Adults should be fed several hours before shipment, and higher than normal mortality is to be expected.
3. One- to three-week old puparia may be shipped in plastic Petri dishes or in canisters, such as the type used commercially to hold 35 mm film, in adequately insulated containers. The Petri dishes or canisters must have air holes, and puparia should be separated from the edge of the container by a layer of cotton wool.

4. When shipping large numbers of puparia, and when delivery will take a long time, care must be taken to provide the puparia with an adequate amount of air in the shipping container.
5. Containers that are open to the environment may result in exposure of the puparia to insecticides or other toxic gases during shipment.

NOTES

1. Collecting and checking blood: The details of the collection procedure, and the equipment needed, will vary depending upon the amount of blood being collected, source of blood, and local slaughtering practices. For small colonies, bovine blood may be obtained from a live animal using sterile technique and equipment. The jugular vein is raised and punctured with a hypodermic needle that is attached to a 1 m long (4 mm diameter) silicone hose leading to a 500 mL Erlenmeyer flask or blood bottle that is about one-quarter filled with 5–8 mm diameter glass beads. Blood is defibrinated by gently swirling the flask or bottle for about 5 min. Larger quantities of blood may be collected from the slit throats of animals at the time they are slaughtered. All equipment used in the process must be clean, dry, and sterile (or at least exposed to 80°C dry heat overnight before use). Blood may be passed through a funnel and tubing into large polyethylene containers that have lids fitted with paddles to defibrinate the blood. Depending upon local circumstances, the paddles should be attached to either hand- or electric-powered drills so that they may be turned gently to defibrinate the blood. (As an alternative to defibrination, sodium heparin [800–1000 IU/L of blood] may be added to the container before blood is collected.) If tradition requires that the animals are slaughtered on the ground, blood may be collected into polyethylene buckets (that have been at 80°C overnight) and transferred immediately to the containers for defibrination. Blood that is collected under clean, but not necessarily sterile, conditions should be transferred to clean heat-treated polyethylene containers of a suitable size and microbially decontaminated by exposure to 1.0–1.5 kGy gamma-irradiation and then frozen.
2. Control of blood quality: A microbiological test and a bioassay are run to determine that each batch of blood is sterile and free of other contaminants, such as insecticides. It is essential that each batch be clearly labelled and that adequate records of the tests be maintained. As a further precaution, batches of blood that have been cleared for use should be stored in a different freezer than batches that have not yet been cleared. Unsatisfactory batches should be disposed of immediately. The microbiological test is conducted by placing 1 mL of defibrinated blood into a sterile Petri dish and adding autoclave nutrient agar (that has cooled to 35°C) to the dish. Two bacteriological controls must also be run. To check that the agar is sterile, use boiled, distilled water in place of blood. As a check on the sterility of the hood in which the blood was transferred to storage vials, an agar plate is left open in the hood for 1 min. The petri dishes are examined after 48–72 h at 25–30°C. The bioassay is conducted by testing samples of each batch of blood for its ability to support a test population of mated females for 25 days. At least 500 mL of the blood is thawed and the diet that is to be used for feeding flies (bovine blood or a mixture of bovine and porcine blood) is prepared. The blood is divid-

ed into 20 mL aliquots (polyethylene scintillation vials are appropriate) and treated with 1–1.5 kGy gamma-irradiation. A bacteriological test is run on each aliquot and the aliquots are stored frozen until use, but are retained only if the bacteriological test is negative. Two cages, each containing 15 teneral females are fed test blood using the standard colony procedures. The females are mated at the appropriate age (below). Mortality is checked each working day, and the apparent cause of death is recorded. The tray on which the flies are held is checked under a binocular microscope on days 10, 15, 20 and 25 for aborted eggs or larvae. Puparia are collected, counted, weighed, and assigned to a puparial class (Table 5.1). Females surviving 25 days are examined for mating scars (seen in members of the *palpalis* group) and dissected to determine insemination, uterine contents, condition of the follicle next in sequence to ovulate, and any reproductive abnormalities (such as oviduct blockage). The data are used to calculate an empirically based quality factor (QF) using the following formula:

$$QF = [FS25 + PT + 0.3(PB) + 0.4(PC) + 0.5(PD) + 0.6(PE) + 0.3(IE) + 0.6(IL) - 0.2(PA) - 0.5\,(AB) - BO] / [FS25 + FS18]$$

where FS25 and FS18 are the numbers of females alive on days 25 and 18 respectively, PT is the total number of puparia produced, PA, PB, PC, PD, and PE are the number of puparia in classes A to E (as defined in Table 5.1), IE and IL, are the numbers of inseminated FS25 females in the early or late stages of pregnancy respectively, and BO is the number of inseminated FS25 females with blocked oviducts. AB is the number of inseminated FS25 females that aborted, i.e. the uterus is empty and the follicle next in sequence to ovulate is not mature. We recommend that during early stages of colony maintenance, a QF value of 1.0 be accepted as indicating that a diet is satisfactory. For some species it may be possible, with experience on about 50 batches of diet, to omit the dissections at day 25 and amend the QF formula and the value used to accept a diet.

Quality control of colonized tsetse – phenotypic measures of quality

1. Puparial weight: This parameter is a good overall indicator of the effectiveness of colony maintenance. Puparia formed during the previous 24 h are collected

Table 5.1 Puparial size classes for various species of tsetse

Species	*Puparial weight**	*Weight ranges for class sizes* A	B	C	D	E
G. tachinoides	17.3 ± 1.9	<14	14 <17	17 <19	19 <21	>21
G. p. palpalis	30.9 ± 3.8	<21	21 <27	27 <31	31 <35	>35
G. f. fuscipes	32.8 ± 3.7	<23	23 <29	29 <33	33 <37	>37
G. austeni	23.1 ± 3.6	<18	18 <21	21 <24	24 <27	>27
G. pallidipes	35.9 ± 3.9	<29	29 <33	33 <37	37 <41	>41
G. brevipalpis	64.4 ± 5.7	<56	56 <61	61 <66	66 <71	>71

* Weight in mg ± SD.

in the morning and weighed in the afternoon. If there are fewer than 50 puparia produced per day, these may be weighed individually. For larger colonies, puparia may be weighed in groups, or the number in each weight class may be determined using a size-sorting machine (Zelger and Russ, 1976). The mean puparial weight should approximate the values given in Table 5.1, and no more than 10% of the puparia should be in weight class A (Table 5.1).

2. Emergence rate: This rate is determined using at least 100 puparia that are not in weight class A and should exceed 85%. If less than this, puparia from which flies did not emerge should be dissected and the state of the contents noted to determine the stage at which excessive mortality occurred. Deaths in the early stages of development are indicated by contents that are almost completely dried, an amorphous liquid, or a formed, but non-pigmented adult. A formed adult with pigmentation confined to the compound eyes indicates death about day 17–20. Adults may also die after being fully formed and pigmented. These results may be compared with environmental records to see if increased mortality can be associated with temperature or humidity fluctuations.
3. Quality control populations: These populations should consist of groups of females that are set up from time to time in order to assure quality. The size of each population and the frequency with which these populations are set up depends upon the size and uses of the colony.
4. Mortality rates: Early mortality rates should be determined when the flies are about 10–14 days old. This indicates mortality during the time when flies are chilled and otherwise manipulated. High mortality may indicate that flies have been chilled for too long, to too low a temperature, or were fed too soon after chilling. Later mortality rates are determined at weekly intervals. These will rise as the flies age. The condition of the dead female should be noted and its cause classed as 'starvation' (abdomen dorsoventrally flattened), 'blood-mortality' (abdomen filled with a black liquid), 'pupa-mortality' (a puparium in the abdomen) or 'other'. All occur randomly and at relatively low frequencies in adult females that are less than 60 days old. An abrupt increase in one or more of the first three categories indicates that specific errors are occurring in colony maintenance. Starvation may occur when: (i) the holding or feeding rooms are too warm or too dry; (ii) flies are exposed to excessive air currents; (iii) the feeding regime is inadequate; or (iv) the feeding surface is unattractive (too hot, too cold or contaminated). 'Blood-mortality' may occur when: (i) the holding room is too humid; (ii) there is excessive ingestion of diet (possibly caused by a nutritionally unsuitable diet); or (iii) *in vitro* blood meal is too hot, contains toxins or is contaminated with bacteria. 'Pupa-mortality' indicates: (i) intoxication of the female during late pregnancy; or (ii) too low humidity in the holding room. In a population of breeding females with a stable age distribution, the overall mortality rate should not exceed 1.2% per day.
5. Fecundity: Fecundity is most readily determined at weekly intervals, but, because of the duration of the reproductive cycle, it is usually expressed as puparia/female/10 days (i.e. 10 × the number of puparia produced during the week divided by 7 × the number of females surviving the week). The numerical value of this parameter varies with the age of the females, and between species. As a general guideline, the first larviposition should occur by the time the females are 18 days old, and an average fecundity of 0.6 puparia/female/10 days is satisfactory to maintain a self-sustaining colony. If fecundity is low, or if there

is a delay in the onset of the reproductive life of females, the environmental conditions in the holding room should be checked. The trays on which females are maintained should be inspected for aborted eggs or larvae. Abortions may be caused by suboptimal holding conditions, excessive crowding, insufficient nutrition, intoxication or bacterial contamination. Low fecundity may be caused by failure of flies to mate (usually detected by visual inspection of cages after males and females have been placed together), or failure of males to inseminate females. The latter may occur if the ratio of males to females in the mating cages is too low, if the males are too young or the females are too old, or if male puparia have been subjected to high temperatures (i.e. >27°C).

6. Genetic measures of quality in laboratory colonies: When discussing aspects of genetic quality in laboratory-reared tsetse, two factors must be borne in mind. First, any genetic bottleneck during initial colonization will, in most cases, lead to loss of the colony. This is because tsetse can tolerate only very small reductions in reproduction before the population crashes. In a rather circular argument, if a tsetse population can be colonized it has, almost by definition, not undergone any significant genetic bottleneck. This is in stark contrast to many other insect pests which do undergo severe genetic bottlenecks when facing laboratory colonization. Second, once colonized, tsetse has another advantage over other insects, in that they will tend to maintain a high level of genetic variability and hence quality. At each stage in its life in the laboratory, tsetse receive nutrition that is identical, or nearly identical, to that received by flies in the field. Adults receive a guaranteed supply of vertebrate blood and the larvae continue to be fed by their mothers. There is no laboratory adaptation to either adult or larval artificial diets, which is in contrast to many other insects where totally artificial diets are provided. For these reasons it is expected that tsetse colonies will not show severe reductions in genetic variability. Electrophoretic enzyme analyses to measure genetic variability in laboratory and field populations have been developed (Gooding, 1990). These techniques indicate that laboratory colonies of tsetse have not exhibited massive losses of genetic variability. Preliminary work with RAPDs has confirmed this observation (Robinson and Gooding, unpub. results). There is very little documented evidence of adaptations to laboratory conditions, probably due to the reasons given above.

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CHAPTER SIX

Care and maintenance of triatomine colonies

Patricia de Azambuja and Eloi S. Garcia

6.1 INTRODUCTION

The family Reduviidae, subfamily Triatominae, includes more than 110 species, several of which are vectors of *Trypanosoma cruzi*, the causative agent of Chagas' disease or American trypanosomiasis. Triatomines are common in the Americas, from the southern USA throughout Latin America, south to Patagonia. Chagas' disease is endemic in 17 countries within this region. Some triatomines are sylvatic and are found in the safety of burrows and nests of wild vertebrates (didelphis, rodents and birds), rocks (especially associated with small rodents), fallen timber, hollow trees, roots, palms and bromeliads. Many species are found in peridomestic locations and in domestic animal houses, whilst others are domestic, occurring inside poor human habitation even when minimum conditions of shelter and food are offered. Domiciliary triatomines are quite opportunistic in their host selection and feed well on humans. They are poor flyers, thus the dispersion of triatomine species (or their eggs) is related to the transportation of man, his furniture, goods or migratory animals.

The most important species of Chagas' disease vectors are *Triatoma infestans, T. dimidiata, T. brasiliensis, T. maculata, T. sordida, Rhodnius prolixus, R. pallescens* and *Panstrongylus megistus* (Lent and Wygodzinsky, 1979). More than 18 million people in Latin America are infected with *T. cruzi* and about 90 million people live in what are considered to be risk zones; consequently, triatomines have been the focus of much research effort aimed at the understanding of both *T. cruzi*-vector interaction and vector control.

6.1.1 Rearing of triatomines

To obtain large numbers of triatomines, a highly standardized feeding procedure, a suitable host blood source, and favourable environmental conditions are needed. Even under these conditions, there are multiple factors involved in rearing, and slight modifications can introduce disturbances in feeding and consequent effects on reproductive potential.

Molecular Biology of Insect Disease Vectors: A methods manual. Edited by J.M. Crampton, C.B. Beard and C. Louis. Published in 1997 by Chapman & Hall. ISBN 0 412 73660 8.

Since all species of triatomines are obligate blood-feeders, all five instars require blood meals for growth and development. Triatomines feed quickly, skilfully and painlessly, at infrequent intervals, taking very large meals of blood, each of which in the preadult stages can be up to 10–12 times as large as the insect itself. Both sexes of the imaginal stage require a blood meal for reproduction.

The insectary is the base for rearing and maintaining triatomines under laboratory conditions. It may consist of one partially darkened room adapted as an incubator or incubators with controlled temperature and humidity in which both larvae and adults are reared. The adapted room is equipped with electric heaters, an automatic thermostatic device, and humidity controls. Temperature and humidity should be monitored continuously. Much information on triatomines (biology, biochemistry, physiology and control, as well as their use in diagnosis – xenodiagnosis) has been obtained through the use of insects reared and maintained in the laboratory. The optimal temperature range for development is 27–30°C. At temperatures greater than 35°C or less than 22°C most insects are adversely affected, showing resultant morphological anomalies, delayed moulting or reproduction, or death. Although humidity requirements are less rigid and can vary depending on the species, 60–85% RH is considered optimum for most triatomine species maintained in the laboratory. Thus, it is necessary to ensure the basic needs, such as temperature, blood meal, humidity and light, for optimal development and survival of different species of triatomines.

6.1.2 Large-scale feeding of triatomines

Since triatomines are haematophagous throughout their entire life cycle, the laboratory rearing method must allow repeated blood meals throughout development. The traditional method involves feeding insects on living hosts, such as rabbits (Buxton, 1930; Hill and Campbell, 1975); sheep (Gardiner and Maddrell, 1972), chicken (Correa, 1954), mice, rats and guinea pigs (Lent and Wygodzinsky, 1979). An alternative method, applicable in some laboratories for rearing of triatomines and/or for special short-term experiments with these insects, involves the use of membrane devices and blood drawn from live mammals either with anticoagulants or defibrinated (Friend and Cartwright, 1963; Gardiner and Maddrell, 1972; Garcia *et al.*, 1984; Pimley and Langley, 1978; Núñes and Lazzari, 1990). However, only Pimley and Langley (1978), Garcia *et al* (1984) and Núñes and Lazzari (1990) have published extensive studies on the successful long-term rearing of triatomines (e.g. *R. prolixus* and *T. infestans*) using membrane feeding.

Details regarding the application of both feeding techniques are given below. It is well understood that each method has its own limitation depending on the unique characteristics of the particular insect vector species of interest. Where and when necessary, the care and maintenance of triatomine colonies should be adjusted to the insect vector species. Temperature, humidity, blood meal source, feeding frequency, meal size, moulting success, size and fecundity of adults, and the time taken for eggs to hatch, as well as other requirements influencing the duration of the life cycle and rate of mortality, are parameters to be considered in order to ensure success in rearing these haematophagous insect vectors in the laboratory.

6.2 MATERIALS

6.2.1 Living host and feeding preparation

1. An apparatus for immobilizing the vertebrate hosts: the particular device selected depends on the size and/or species of the animal utilized for feeding but must firmly hold both the legs (or wings in the case of a chicken) and the body of the host.
2. Suitable vertebrates for feeding of insects: should be in good health; adults are preferable (Notes 1–4).
3. Suitable clean containers for caging starved insects: can be made of plastic or glass and should measure approximately 10 cm in diameter × 15 cm high for 100 insects. The size of the container for larvae depends on either the developmental stage and/or the number of insects to be fed. Entomological soft forceps, adapters to hold position, fixation clamps and supporting stand, are also needed.
4. Materials for containers for maintaining fed insects: nylon gauze cloth, filter paper or blotting paper, rubber bands and large glass or plastic containers. For 200 adults the container should be approximately 25 cm in diameter × 40 cm in height.
5. A maximum–minimum thermometer is needed for temperature monitoring.
6. All of these materials can be obtained commercially. Specific selections depend on cost and availability.

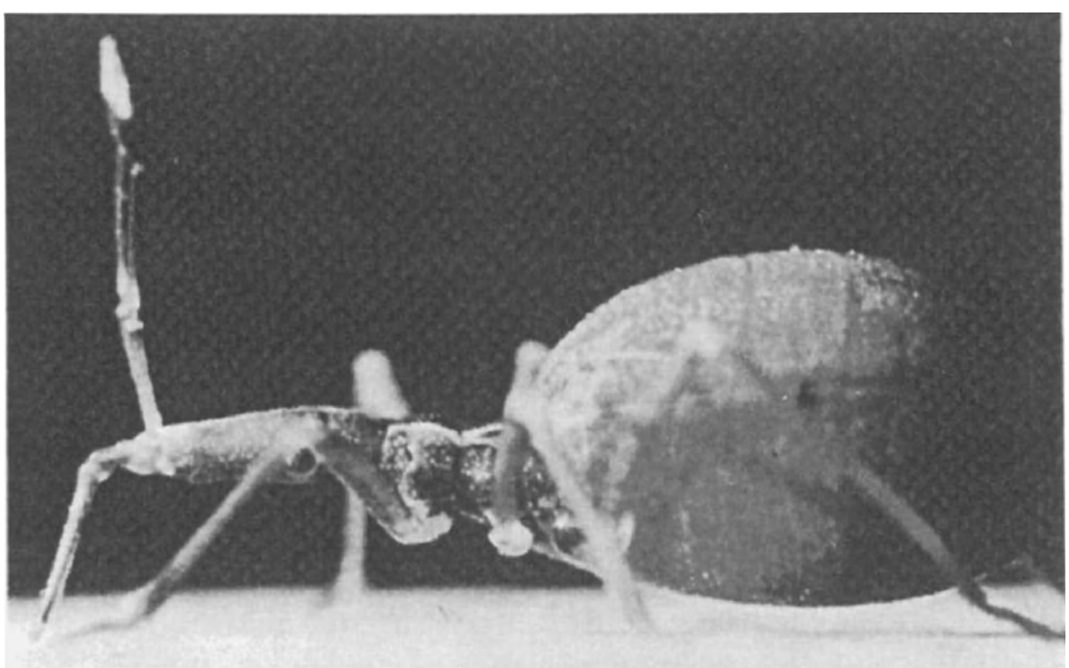


Figure 6.1 A fourth-instar larvae of *Rhodnius prolixus* feeding on a rabbit.

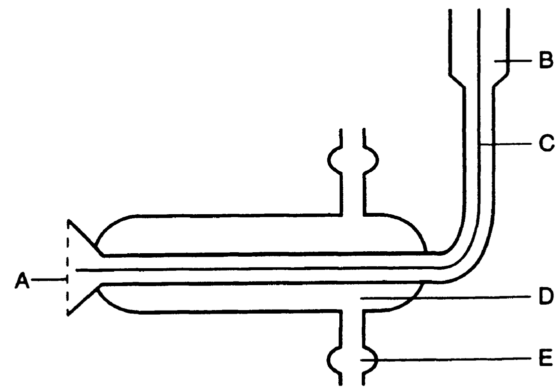


Figure 6.2 Schematic diagram of the membrane feeding apparatus. (A) Feeding membrane; (B) blood chamber; (C) thin polyethylene tube connected within a syringe; (D) heated water chamber; (E) connection of the water chamber with a water bath circulator set.

6.2.2 Membrane device and feeding preparation

1. An artificial feeding device as depicted in Figure 6.2: an L-shaped glass funnel (ranging from 5 to 50 mL in capacity) that contains a heated tube glass which is connected with a water bath circulator set at 37°C. The mouth of the funnel (ranging from 2–5 cm in diameter) is capped with a tightened membrane held in place by a rubber band.
2. Suitable feeding membranes: include thin rubber, latex, surgical gloves, condom, Parafilm and baudruche (a piece of the dry intestine of cattle or sheep). A comparison of the efficiency of different membranes for each triatomine species is recommended.
3. Water bath circulator with thermometer: in order to check that the blood meal is at a constant temperature of 37°C.
4. Blood meal: usually citrated blood (18.4 g glucose, 16.5 g sodium citrate, 6.0 g citric acid, dissolved in re-distilled water to a final volume of 100 mL). To each 100 mL of blood, 1 mL of this solution is added. Heparinized blood (5–8 IU of heparin/mL) or defibrinated blood (using small glass balls) can also be used.

6.3 METHODS

6.3.1 Feeding on living hosts

The following method can be used to feed triatomines at any developmental stage. The production of large numbers of insects will depend exclusively on the number, size and the species of the host used.

1. Vertebrate hosts must be suitably placed in a special compartment for immobilization and rested in a partially darkened room without noise for some minutes.
2. The area of the animal's body over which the container with starved insects will make contact during feeding must be clean (*do not use soap/detergents or any chemicals*) and shaved (*use a suitable razor*).

3. Starved triatomines of any instar or imaginal stage are caged in clean glass or plastic containers and covered with a piece of nylon gauze cloth held in place by a rubber band. Containers with unfed insects are placed at odd angles on the supporting stands with adapters to hold position and fixation clamps to enable very close contact with the host's body.
4. After full gorging (no more than 30 min of feeding) the insects are transferred to large jars containing clean filter or blotting paper vertically folded for easy air flow. Also, filter or blotting paper provides hiding places and facilitates insect circulation (up and down) in the containers; it also absorbs the enormous volume of urine and faeces excreted by the insects.
5. The size of the jar must correlate with both the size of the insect instar and the numbers of individuals. These containers (described above) must comfortably house the number of insects placed in them. Cage number, date, and observations of moulting are recorded in a separate table.
6. The lid of the jar is closed with taped nylon gauze or other thin cloth and the jars are held in racks of the incubator or insectary. Care is taken not to disturb the fed insects before they have moulted. Any manipulation of gorged triatomines may increase mortality (Notes 1–8).

6.3.2 Feeding on membrane devices

Although the rearing of triatomines in the total absence of a live host is not widely used as a routine method, the membrane feeding technique has proven very practical for artificial feeding of triatomines. This method has been used routinely in our laboratory for more than 25 years to produce large numbers of *R. prolixus* (more than 100 000 insects/year) (Notes 9, 10).

1. The tube of the feeding device is filled with blood (Figures 6.2, 6.3 and 6.4), carefully avoiding air bubbles on the feeding membrane, and fixed on a supporting stand by brass fixation clamps. Sometimes to avoid erythrocyte sedimentation, the blood is gently mixed by either pressing the feeding membrane or using a syringe connected within the blood tube through a thin polyethylene tube.
2. Warm the feeding device to 37°C using the water bath circulator connected to the apparatus. Warming the blood is important in order to stimulate orientation of insects to the membrane and to induce probing. Temperatures higher than 39°C act as repellents, however, and lower than 30°C fail to adequately stimulate probing and subsequent engorging.
3. Starved insects, contained as described in Section 6.3.1, are placed on supporting stands and held in position with clamps in order to hold them securely in contact with the membrane of the feeding apparatus.
4. After feeding is complete (usually around 30 min) the insects are transferred to large jars containing clean folded filter or blotting paper and maintained as described in 3.1.
5. By using ten feeding devices of 50 mL capacity, it is possible within a few days to feed approximately 20 000 insects with a minimum problem and risk of escape.
6. The feeding device is easy to clean and, if necessary, to sterilize (Notes 11–17).

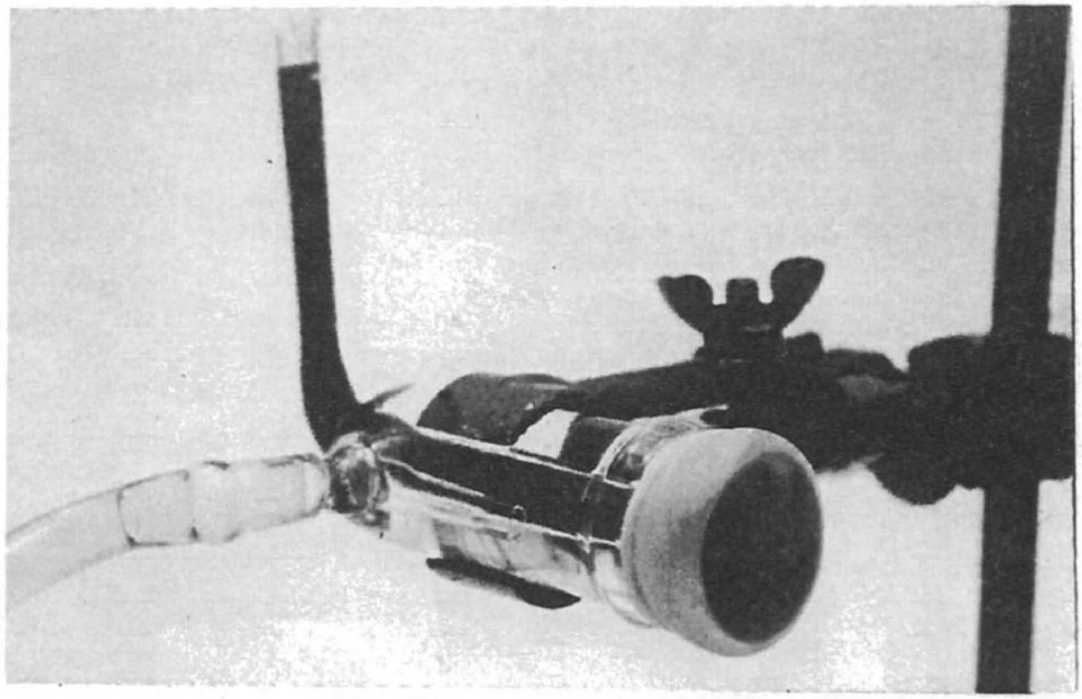


Figure 6.3 A typical membrane feeding apparatus filled with blood.

NOTES

A number of additional factors are described below which are very important in caring for and maintaining large colonies of insects in the laboratory.

1. Nutritional and health conditions of the vertebrate hosts must be continuously monitored. Do not feed triatomines on weak and/or sick animals and avoid the use of immature animals.
2. Avoid the treatment of living hosts with drugs against internal and external parasites, food with antibiotics or contamination with insecticide.
3. Avoid very active living hosts that cannot be properly constrained because they can kill some insects during feeding.
4. Host species used for feeding triatomines can affect the growth and reproduction of the insects. For example, we have observed that *R. prolixus* and *T. infestans* fed on cows or sheep for prolonged periods of time showed dramatic delays in their development, decreased reproduction, and higher mortality rates.
5. Avoid a high number of starved insects in the feeding containers. Ensure that insects that complete their feeding early do not disturb those that are still feeding.
6. Overcrowding of engorged insects in the containers results in increased mortality and less than satisfactory development and reproduction.
7. Humidity control is necessary to optimize rearing conditions. Since triatomines, after consuming a gigantic blood meal, excrete large quantities of ingested fluid

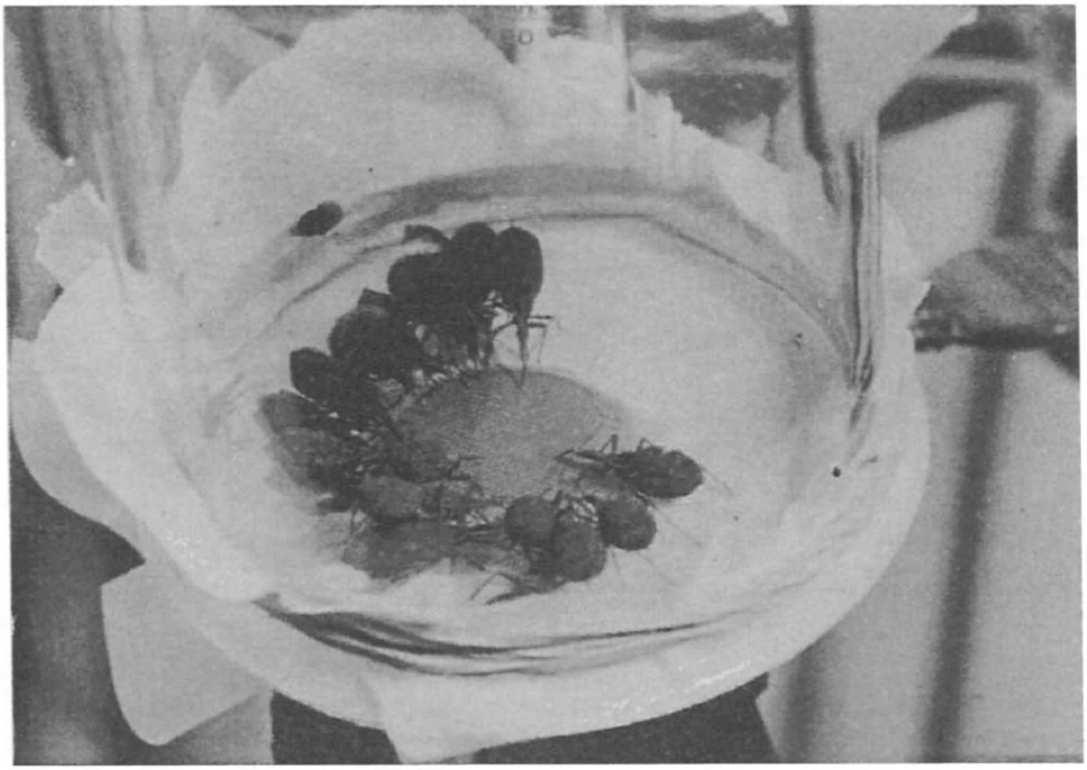


Figure 6.4 Fifth-instar larvae of *Rhodnius prolixus* feeding on a membrane feeder.

(urine and faeces), within a few hours the humidity increases and this can cause a high mortality in the colony.

8. Cleaning the containers and changing filter or blotting papers must always be performed before feeding, when the insects are starved and separated for feeding.
9. For some triatomine species, e.g. *T. infestans,* it may be necessary to provide additional feeding stimulants such as CO_2, animal odours, or by the addition of ATP as a phagostimulant to the blood meal.
10. The frequency of feeding using both methods (Sections 6.3.1 and 6.3.2) depends on a combination of rearing temperature and humidity, as well as on the particular triatomine species. Newly hatched or ecdysed larvae do not feed well during the first 3–4 days. Our experience with *R. prolixus* large-scale rearing shows that the first instar larvae feed to repletion 7 days after hatching. The other instar larvae fed once in 3–4 weeks, following rotation feeding which gives effective time for these insects to digest their blood meal, to moult, and to be ready for the next feeding without excessive starvation which may cause an increase in the mortality rate. Insects which do not feed and/or moult are given a second opportunity to feed and moult before discarding. To maintain a triatomine colony the adults should be fed at intervals of 2–3 weeks and any eggs removed immediately prior to insect feeding. The incubation period lasts about 14–17 days at 28°C. For other triatomine species the frequency of feeding and the incubation period for eggs are more variable and must be standardized. Some species need more than one blood meal to ecdysis from one to the next instar. The care and

maintenance of triatomine instars and adults are optimized when each instar larvae and adults are maintained in separate jars.

11. Use only blood donors with good nutritional and health conditions. Triatomines are extremely sensitive to some deleterious substances contained in the food used to feed the hosts that provide the blood.
12. Blood conservation time and its contamination with microorganisms, temperature of blood conservation and haemolysis can affect both blood-feeding and consequent survival after the feed. Usually fresh blood with no more than 24 h of storage at 5°C is used. Some triatomine species do not feed well on refrigerated blood (Azambuja and Garcia, unpublished data).
13. Some anticoagulants can be problematical in the rearing of triatomines through membrane feeders. Oxalate or fluoride solutions must be avoided because they produce high mortality in the colony. Citrated blood has been shown to be more suitable than heparinized blood (Azambuja and Garcia, unpublished data).
14. Host blood source can affect both the development and reproductive performances of triatomines. For instance, *R. prolixus* reared from eggs to adults on varieties of blood, including human, rabbit, chicken, sheep and horse blood sources in comparative studies, show dramatic differences in biological cycle, size, and fecundity. Larvae develop faster with a higher number of them attaining adulthood, and the resulting females lay more eggs when they are membrane-fed with human and rabbit blood. The inefficiency of chicken and horse blood sources is manifested in a decrease in blood meal ingestion and a resultant reduction in development, size and reproduction after some years of rearing. The deleterious effects of sheep blood in long-term rearing are observed by an unacceptably high mortality, poor ecdysis and a drastic reduction in egg production (Gardiner and Maddrell, 1972; Gomes *et al.*, 1990).
15. Do not wash the feeding membranes with soap/detergents or any chemicals, clean them with either water or physiological saline only.
16. Avoid air bubbles that rise and lie directly under the feeding membrane when the feeding devices are in use.
17. Further details regarding the feeding and maintaining triatomines after gorging are included in the publications listed below.

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Part Two
Experimental Infection of Insect Vectors

CHAPTER SEVEN

Infection of mosquitoes with rodent malaria

Robert E. Sinden

7.1 INTRODUCTION

The purpose of this introduction is to describe in outline only the salient aspects of the biology of *Plasmodium* and the mosquito vector that are required to understand the rationale of the protocols given. For a more detailed analysis the reader is directed to the comprehensive works on the subject published by Shute and Maryon (1966), Vanderberg and Gwadz (1980), Sinden (1984) and Carter and Graves (1988). An in-depth review of the complex factors regulating the transmission of *Plasmodium* to the mosquito (Sinden *et al.*, 1996) may also be useful.

The protocols described relate solely to laboratory procedures for the transmission of rodent malarial parasites to their mosquito vectors, and for monitoring such infections. Protocols on how to prepare the sporogenic stages of parasite for subsequent molecular analysis are too varied and specialized for inclusion in a general description of infection techniques.

7.1.1 The biology of transmission of rodent malarias to the mosquito vector

(a) The gametocyte

Malaria is transmitted to the mosquito solely by the sexual stages (gametocytes). The intra-erythrocytic male and female parasites are arrested at the G_0 phase of the cell cycle and circulate in the peripheral bloodstream either until the death of the host red blood cell (RBC) or until they are ingested by a female anopheline mosquito. Individual gametocytes typically take 22–28 h to mature, and persist for another 1–2 days in the circulation. When observing the pattern of gametocytaemia in the infected rodent host there are marked differences in distribution over time between the different parasite species. These differences have a direct impact on the pattern of infectivity to the mosquito vectors, and therefore upon the management of the infection in the laboratory. Simply stated there are those parasites that produce a rapidly fulminating and often lethal infection (e.g. *P. berghei* in the T.O. mouse) and those that produce a low-grade or non-lethal parasitaemia which is subsequently cleared (e.g. *P. chabaudi* in the mouse). In the first group, gametocyte numbers represent a

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constant fraction of the total parasites, there is an early rise in infectivity followed by an abrupt cessation of infection despite the rapidly increasing gametocyte numbers. This blockade of infectivity is not due to a specific immune response, but certainly relates to an interaction between the asexual parasites and host physiology (below). In contrast in those non-lethal parasite/host combinations that may fail to reach high parasitaemia, infectivity may be sustained for extended periods and in some species (e.g. *P. chabaudi*) (Walliker *et al.*, 1975).

Infectivity of viable gametocytes to the mosquito vector is modulated by a wide range of factors. Antibodies develop against surface antigens of the gametocyte some of which are shared with the gamete, zygote and ookinete. Such antibodies will block fertilization, lyse the extra cellular gametes and zygotes in the mosquito blood meal through complement dependent mechanisms, or block the motility and invasive activity of the ookinete preventing its passage through the midgut wall. It has been shown that SCID mice, which fail to produce B and T cells and therefore antibodies, effectively suppress gametocyte infectivity of *P. berghei* from day 5; clearly therefore other factors also suppress transmission to the mosquito.

Work on *P. vivax, P. cynomolgi* and *P. yoelii* has suggested that at the time of schizogony the parasite releases factors which either directly or indirectly (*via* TNF and IFNγ) mediate the release of products inhibited by L-NMMA (i.e. nitric oxide), which in turn either directly or indirectly kill the infectious gametocyte (Mendis *et al.*, 1990; Naotunne *et al.*, 1993; Motard *et al.*, 1993). Recently Prada *et al.* (1995) have suggested that haemozoin is a powerful inducer of TNF production.

In *P. berghei* the serum mediated inhibition of infection seen from day 5 onward is essentially independent of IFNγ, TNFγ, neutrophils and nitric oxide, but can partially be reversed by the addition of bicarbonate to the serum (to achieve final concentrations of 20 mM). This correlates with a decline in serum bicarbonate concentration with the increasing parasitaemia which is brought about by the lactic acidosis induced by the expanding parasite population.

Parasites that display a sustained or even 'late' infectious period such as *P. chabaudi* or *P. y. nigeriensis* are, in general, less virulent in the hosts harbouring such infections – again nurturing the idea that the responses of the host to the 'virulence' of the asexual parasite are key factors regulating infectivity.

These observations raise the simple practical point that to obtain good transmission of the more virulent parasites it is necessary to feed mosquitoes directly on hosts before any inhibitory responses arise, normally in the first 5 days of infection. It is always with great amusement that I recall Professor Garnham's response to my naive question 'When is the best day to feed the mosquitoes?', 'On the day *before* you see the first gametocyte' was the typically prophetic and mischievous reply! If it is essential to feed on virulent infections older than 5 days the use of membrane feeder systems in which the preparation of the parasitized erythrocytes includes replacement of the inhibitory serum with normal serum, or supplementation with bicarbonate, is strongly recommended.

Recognizing this recent data showing that both immune and non-immune serum factors can reduce infectivity to the mosquito, it is now perhaps less surprising to record that concomitant infections such as Eperythrozoon have been shown to inhibit the development of the malarial parasite (Voller and Bidwell, 1968).

Landau and Boulard (1978) have summarized the practical aspects of natural infections of laboratory hosts with rodent malarial parasites, including the resulting infectious periods to mosquitoes and conditions for the maintenance of the vectors to

achieve transmission of the parasites. Her table (reproduced here as Table 7.1) records typically the time of infection from a blood-induced parasitaemia. Sporozoite induced infections obviously are more delayed due to the intervention of the pre-erythrocytic cycle which typically takes 44–60 h, and results in a lower initial parasitaemia. Sporozoite induced infections of 'lethal' parasites often contrast with blood-induced infections in that the outcome may not be lethal.

For parasite management in the laboratory, use of blood passage is advised for one practical reason only, that a high gametocytaemia can be reached before any significant inhibitory host response is generated. One should be aware that a serious drawback in the use of blood passage is the rapid loss of infectivity of parasite isolates with repeated passage (Birago *et al.*, 1982; Huff *et al.*, 1958; Vanderberg and Gwadz, 1980). This has been shown to be due to chromosomal breakages resulting in the loss of genes essential to the production of gametocytes (Birago *et al.*, 1982; Janse *et al.*, 1991; Ponzi *et al.*, 1990; Frontali, 1994). These genes are, on current evidence, clustered on chromosome 5 of *P. berghei* (Janse *et al.*, 1989) (and possibly also in other rodent parasites, Barker *pers. comm.*). In our laboratory, the parasite most transmitted (*P. berghei* ANKA clone 2.34) originally produced infectious gametocytes in useful numbers for at least eight mechanical passages (each of 1–3 weeks duration). However, over a period of 15 years we now find only 2–4 mechanical passages are possible before it is necessary to reselect for an entire genome by mosquito passage. It is obvious from this statement that a key factor in the simple management of malarial parasites is the provision of highly replicated cryopreserved parasitized erythrocytes following sporozoite inoculation (P_0) or following first blood passage (P_1). We note with interest reports of cryopreservation of sporozoites (Leef *et al.*, 1981), but have no personal experience of this procedure.

(b) Gametes

Gametes are induced to form in the mosquito midgut. Gametogenesis is triggered by two factors, a drop in temperature from 38°C to 20–26°C, and the exposure either to gametocyte activating factors (previously termed MEF or mosquito exflagellation factor), or to a rise in pH_0 (Sinden *et al.*, 1996). Thus, if for any reason it is necessary to manipulate gametocytes before infecting mosquitoes it is essential to avoid reproducing these conditions. Routinely this is achieved by assiduously maintaining the temperature of infected blood at 37–38°C and avoiding exposure to air which results in a rise in pH_0. Parasites are able to endure these conditions only for relatively short periods, e.g. 30 min maximum.

We find that monitoring exflagellation *in vitro* is a sensitive assay by which to determine the *potential* infectivity of gametocyte carriers to mosquitoes. However, a positive exflagellation assay does not guarantee infection (because many factors that inhibit infection are diluted in the assay, but not in the blood meal). For routine exflagellation assays we use as a diluent either serum, previously adjusted to pH 8.5 (by exposure to air), or buffered RPMI 1640 pH 8.4, at 20–22°C.

It is on occasion necessary to monitor directly exflagellation as it occurs in the mosquito midgut. This is easily done, but does require rapid dissection of the mosquitoes as the process is usually initiated within 10 min or less following uptake of the blood-feed. A simple but less useful screen can be made by collecting the droplets of blood excreted by the females during feeding. This, if observed undiluted by phase or interference microscopy, can contain exflagellating microgametocytes.

Table 7.1 Optimal conditions to ensure cyclical transmission of rodent *Plasmodium*. Modified from Landau and Boulard (1978)

Parasite	*Suitable gametocyte carriers other than natural hosts*	*Best days of blood-induced infections for mosquitoes to be fed*	*Suitable laboratory mosquitoes*	*Unsuitable laboratory mosquitoes*	*Best temperatures for sporogony (°C)*	*Days glands first invaded*
P. berghei (Katanga)	Some strains of white mice, white rats, hamsters, muskrats	4–5	*A.stephensi* *A.l.atroparvus* *A.annulipes* *A.gambiae A?B* *A.aztecus* *A.quadrimaculatus*	*A.albimanus* *Aedes aegypti* *Culex salinarius*	19–21	14
P.y.yoelii (CAR)	Mice, white rats, hamsters	4–5	*A.stephensi* *A.sundaicus*	*A.l.atroparvus* *A.b.balabacensis* *A.quadrimaculatus*	24	9
P.y.killicki (Brazzaville)	Mice, white rats	3–6	*A.stephensi*		22–24	10
P.n.nigeriensis (Nigeria)	Mice, white rats	3–10	*A.stephensi*		24	9
P.v.vinckei	Splenectomized or hairless mice	4–8	*A.stephensi*	*A.gambiae* *A.albimanus* *A.quadrimaculatus* *A.l.atroparvus*	20–21	13
P.v.lentum (Brazzaville)	*Hybomys univittatus*	5	*A.stephensi*		24–25	10
P.v.brucechwatti (Nigeria)	Mice, multimammate rats	10	*A.stephensi*	*A.stephensi** *A.l.atroparvus** *A.quadrimaculatus** *A.gambiae*†	24–26	13
P.v.petteri (CAR)	Mice		*A.stephensi*		24–26	11
P.c.chabaudi (CAR)	Mice, multimammate rats	10–18	*A.stephensi*	*A.aztecus* *A.freeborni* *A.sundaicus*	26	10
P.c.adami (Brazzaville)	Mice		*A.stephensi*		24–26	11

* At 23–25°C sporozoites which were never infective first invaded the salivary glands of these mosquitoes on the twelfth day of infection.
† Insusceptible.

(c) The zygote and ookinete

Fertilization usually takes place within an hour of induction of gametogenesis both *in vivo* and *in vitro*. The zygotes are formed with relatively low efficiency and ookinete numbers often represent only 10–20% of the macrogametocytes used to initiate a culture or infection. Zygotes are poorly infective (usually non-infective) to the mosquito vector 7–12 h after initiation of culture. This period in *P. gallinaceum* corresponds to a period when the parasite is sensitive to complement and corresponds to a time of significant changes in the parasite surface coat (Grotendorst and Carter, 1987; Grotendorst *et al.* 1986; Kaushal *et al.* 1983; Kaushal and Carter, 1984). Meiosis occurs in the zygote and retort-form ookinete.

Early development of the zygote includes the assembly of the apical complex which buds from the surface of the spherical zygote. This extends progressively until the whole cell is integrated into the ookinete which is surrounded by a plasmalemma dominated by two surface proteins of 21 and 28 kDa. Beneath this lie the inner two pellicular layers, under which lie ±60 equally distributed microtubules coiling down the length of the cell. An extensive rhoptry and microneme vesicle complex develops, and the mature banana shaped ookinete becomes slowly motile. About one in 70 macrogametocytes forms an ookinete *in vivo*. The ookinetes of *P. gallinaceum* and *P. falciparum* traverse the peritrophic layer by secreting a pro-chitinase enzyme which is activated by the trypsin enzymes of the mosquito midgut. Thereafter the ookinete invades the midgut epithelium in a manner highly reminiscent of the merozoite invading the erythrocyte or the sporozoite invading the hepatocyte.

In refractory mosquitoes the ookinete can be melanized in the parasitophorous vacuole as it traverses the midgut epithelium (Collins *et al.*, 1986). Vernick *et al.* (1995) have also described a different refractory phenotype in which ookinetes of *P. gallinaceum* are lysed in the midgut wall of *An. gambiae*. We have seen melanized ookinetes in *P. berghei/An. stephensi* infections but these are rare. We have not noted ookinete lysis in the midgut wall, although ookinetes are degraded in the blood meal from 18 h onward.

Just as multiple invasion of the erythrocyte by the merozoite is believed to lyse the host cell, massive invasion of the mosquito midgut by ookinetes can also be pathological – causing extensive breakdown of the midgut wall and mosquito death. This can be a significant problem when cultured ookinetes rather than gametocytes are used to induce infections. The large numbers of ookinetes that can be produced *in vitro* are nonetheless *very* reliable sources of infection. This should not be too surprising as most of the naturally occurring inhibitors of gametocyte infectivity appear to inhibit gametogenesis and not subsequent events (Sinden *et al.*, 1996). Damage incurred in the mosquito midgut wall due to ookinete invasion is described as a critical factor in the establishment of other pathological infections in the insect, including *Serratia marcesens* (Seitz *et al.*, 1987). Other parasites considered to be detrimental to infection include *Nosema* (Hulls, 1972; Margos *et al.*, 1992), although this parasite is more a problem to the survival of a laboratory colony rather than parasites in individual mosquitoes! In our hard won experience we have found the only solution to *Nosema* infections is to destroy the colony and all minor equipment that cannot be sterilized and sterilize the entire insectary with formaldehyde. Use of benomyl or other proprietary agents against *Nosema* we have found to be useless, as is washing eggs in benzylkonium chloride.

(d) The oocyst

Approximately 1/3500 gametocytes produce an oocyst, although this efficiency rises with decreasing gametocyte (or asexual?) density in the blood meal. The oocyst forms

beneath the basal lamina of the midgut but outside the midgut epithelium basement membrane. It is therefore extracellular. In rodent infections at 21–26°C most ookinetes reach the basal lamina within 24 h. Here they rapidly differentiate, becoming spherical and resorb the apical complex into the cytoplasm of the enlarging oocyst. Initially, 2–3 μm in diameter, the oocyst rapidly increases in size to some 35–75 μm on the 14th to 20th day of infection. The oocyst is readily recognized by phase or interference contrast microscopy from the 5th day of infection. However, at earlier times it is sometimes necessary to resort to mercurochrome staining to count the parasites.

Oocyst counts are the usual method to determine the success of malaria transmission to the mosquito, though epidemiologically this is not the most relevant observation (salivary gland sporozoite counts are more relevant). Oocyst infections may be recorded by intensity or prevalence or a combination of both. Medley *et al.* (1993) have shown clearly the numerical relationship between intensity and prevalence and conclude that either index is a useful measurement. The intensity of infection is usually recorded as the geometric mean number of oocysts per mosquito. Geometric means are preferable because this accounts for the strong negative binomial distribution of oocysts in a population of mosquitoes. Prevalence rates are, however, much easier to record and clearly have a role to play in large scale studies.

Oocysts rupture from day 9 onward depending on the rate of maturation (Table 7.1). Highly crowded oocysts, as often occur in laboratory infections (where over 1000 oocysts can be produced), do not mature at a uniform rate and numerous aberrant (dying) oocysts are produced with highly pycnotic nuclei and vacuolated cytoplasms. Some of these oocysts may, in older infections, become heavily melanized (Collins *et al.*, 1986; Paskewitz *et al.*, 1988) forming Ross's black spores.

Sporozoites are released from the oocyst into the haemocoele, but these sporozoites are not immediately infectious to rodent hosts; though they are able to invade the salivary glands (Vanderberg, 1975).

(e) The sporozoite

Sporozoites released into the haemocoele can be harvested easily using the expedient of removing mosquito heads and separating the thorax and abdomen. Sporozoites are then removed from the mosquito body segments by centrifugation, these sporozoites are poorly infectious to mice.

Sporozoites accumulate in the salivary glands from day 9 onwards (Table 7.1). These sporozoites are infectious to rodent hosts, and differentially infectious to different inbred lines and species (see below). Salivary gland burdens are confined to the distal median lobe and the lateral lobes. These can be very heavy; in our hands *P. berghei* has over a period of 20 years averaged 11 000 salivary gland sporozoites recovered per mosquito. Recognizing that only 20–50 sporozoites are delivered by each bite, the relevance of such high sporozoite numbers to transmission in the field is unclear and has not been studied in adequate detail. Sporozoite infections are, on a small scale, best determined by simple haemocytometry, but on a larger scale radioimmunoassay (Zavala *et al.* 1985) or ELISA (Wirtz *et al.* 1985) are much more appropriate, though less accurate. Any antibody assay using the dominant sporozoite surface coat protein (CSP) as the probe should take into account that CSP expression varies between the oocyst and salivary gland sporozoites, and that CSP is shed into the haemocoele. It is therefore only a crude estimate of the sporozoite pop-

ulation in a mosquito homogenate. Haemocytometry, although tedious, does count sporozoites!

Not all sporozoites reach the salivary glands; estimates vary from <1–11%. In our experience the average oocyst burden we have produced over a 20 year period with *P. berghei* is 70 oocysts, each oocyst produces 8000 sporozoites and yet only 11 000 sporozoites are in the glands, i.e. 2% of oocyst sporozoites reach the glands. Not infrequently laboratories have periods when normally successful vector/parasite combinations, e.g. *P. berghei/An. stephensi,* temporarily fail to produce salivary gland infections despite apparently normal oocyst burdens. The reasons for this are still not known, and do not appear to relate to possible arborviral infections, but could easily be explained by other stresses or damage to the insects resulting in the activation of the mosquito's immune system which then kills the sporozoites whilst crossing the haemocoele.

(f) The mosquito

Details on the breeding of *Anopheles* mosquito vectors is covered in Chapter 1. We routinely use *An. stephensi* as the vector for any of the rodent malarial parasites. Adult mosquitoes are collected by vacuum aspiration from covered pupal bowls, or pupae are aspirated and transferred to clean water. Adults are stored in 30 cm^3 cages at 26°C 80% RH for 4 days prior to infection and are fed 8% fructose 0.05% PABA *ad libidum* (Jacobs, 1964). 24 h before feeding, the fructose solution is removed to encourage feeding. Shorter starvation often results in low numbers of mosquitoes feeding, longer starvation results in unacceptable losses particularly in the older mosquitoes. The body weight of the mosquito reduces significantly upon starvation, which makes studies on the relationship between body weight and blood meal weight difficult to measure accurately.

Feeding can be achieved successfully at any time of day, but is enhanced if the mosquitoes are left undisturbed and in dusk/dark conditions. We find no influence of the circadian cycle upon the mouse host relevant to infectivity of *P. berghei,* although others have suggested that *P. yoelii* infected mice are more infectious in the late evening (Targett, *pers. comm.*; Verhave, *pers. comm.*).

Mosquito density can be an important factor, particularly if the feed is not attractive to the vector, e.g. membrane feeding. For this technique, we put only 50 mosquitoes in a 500 mL container and feed on a 1.5 cm diameter membrane feeder. In contrast, many thousands of mosquitoes will successfully and rapidly feed from a 30 cm^3 cage directly upon a (large) rodent host despite the crowding of the insects during engorgement.

Following feeding the fully engorged female mosquito is very delicate and ideally should not be handled for 12–24 h. If it is necessary to segregate fed and unfed females, it is essential to handle only the *un*feds. We routinely poot (aspirate) mosquitoes and use neither CO_2 anaesthesia nor cold treatment in the first hour after feeding as both could affect the processes of gametogenesis (see above). After 12–24 h of infection CO_2 anaesthesia is both safe and very simple to use; anaesthetized insects can be kept immobile on ice or under intermittent exposure to CO_2. Similarly, if a cold room is available, cages can now be opened and the quiescent mosquitoes sorted with fine tweezers. Mosquitoes, and the malarial infection, benefit enormously if the infected mosquitoes are given a second blood meal between 3 and 6 days after infection (Ponnudurai *et al.*, 1989; Ranawaka *et al.*, 1993). This not only enhances the survival of the mosquitoes, but increases numbers of sporozoites pro-

duced. Since this occurs without superinfection, it suggests the availability of nutrients is a critical factor in oocyst development – which is consistent with the observation on the impact of oocyst density on the speed of parasite maturation.

(g) The rodent hosts

In the context of this chapter rodent hosts serve simply as delivery systems for gametocytes, or receptacles for sporozoites. Animals with low level non-lethal parasitaemias sustain infectivity longer than those with a fulminating acute infections. Infections should be managed in the light of these observations; however use of sporontocidal drugs such as pyrimethamine should obviously be avoided in such management measures. There is considerable evidence to show that where a parasite will infect a range of laboratory hosts the choice of species or inbred line can influence infectivity to the mosquitoes significantly. For *P. berghei* clone 2.34 we find in the T.O. mouse and Balb/c mouse infectivity rises for the first 5 days then rapidly declines to zero, whereas in the old rat a 'chronic' parasitaemia remains infectious for at least 8 days. Following splenectomy the young rat is, in some laboratories, considered a preferable host for induction of gametocytaemia (Cornelissen and Walliker, 1985). Others consider the hamster a preferred host for the induction of *P. berghei* gametocytes (Vanderberg and Gwadz, 1980). *Thamnomys* is a difficult host to maintain and offers few advantages for gametocyte production. It is however exquisitely sensitive to sporozoite infection, whereas the laboratory mouse is not – (infective sporozoite inocula are $>10^4$, 10 and 4 for Balb/c, C57 mice and *Thamnomys* respectively).

We find no advantage in keeping our rodents on an inverted light cycle though for those species where a diurnal pattern of infectivity is suspected (e.g. *P. yoelii*) this could be very useful for day-to-day management.

It is necessary to anaesthetize rodents for blood-feeding, blood collection and sporozoite infection by bite and barbiturates, e.g. Saggatal®, can be used. However, we find the drop in body temperature induced is detrimental to the vigour with which the mosquitoes are attracted, and no longer use this reagent. Currently for all our animals we use a mixture of Rompun® (a muscle relaxant) and Ketamine (Vetelar®, an anaesthetic) which is well tolerated on repeated usage and can have a very long 'knock-out' period followed by a rapid recovery (the animals however can become hyperactive immediately prior to onset of anaesthesia).

7.2 MATERIALS

7.2.1 Parasite passage in mice

1. 1.2 mg/mL solution of sterile phenyl hydrazine.

7.2.2 Materials for cryopreservation

1. Preservative-free heparin (Sigma).
2. Glycerol: 30% v/v in phosphate buffered saline (PBS).
3. Dimethyl sulfoxide (DMSO).
4. Cryotubes.

5. Freezer: –80°C is required, a programmable freezer is optimal (Planar Bench top freezer R204, Planar Products Ltd., Windmill Road, Sunbury on Thames, Middlesex, TW16 7HD, UK).
6. Liquid nitrogen/liquid nitrogen freezer.
7. Water bath.
8. Syringes: 1 mL and 5 mL.
9. 21 G needles.

7.2.3 Materials for mosquito preparation

1. Fresh water aquarium salts: distilled water containing 0.1 g/L 'Pond guardian tonic salts' (Interpret Ltd., Dorking, Surrey RH4 3YX, UK).
2. Mosquito stock cages: we use 30 cm^3 cages (Various; e.g. Bioquip Products, 17803 La Salle Avenue, Gardena, CA 90248-3602, USA) for 10^3 females; 10 cm^3 for 200–1000 females, and 500 mL Nalgene containers for 50 mosquitoes (Nalge Company, Sybron Corporation, Box 20365, Rochester NY 14602-0365, USA.). The sides of the Nalgene containers are roughened with sandpaper, and two holes punched in the lid, one 3–5 cm diameter netting covered for feeding, the other with a 'crossed' rubber dam to take a small tube with a cotton wool or cigarette filter plug to hold the feeding solution. The bottom of the cage is covered with filter paper to absorb spilt liquids (fructose or blood) which otherwise promote fungal infection.
3. Feeding solution: fructose/PABA solution (0.5 g Para-amino-benzoic acid [PABA] and 80 g fructose per L distilled water); autoclave or filter sterilize for storage.
4. Alternative sources of sugar are fruit (surface sterilize by boiling) or diluted unpurified plant sugars (Maple syrup, corn (Karo) syrup etc.). Provision of PABA is considered by some to enhance mosquito infections of rodent parasites (Jacobs, 1964).
5. Mechanical aspirator (Hausers Machine Works, Toms River, New Jersey, NJ 08753, USA).

7.2.4 Materials for monitoring parasitaemia

1. Absolute methanol.
2. Giemsa stain (British Drug House (BDH) R.66).
3. Giemsa buffer: 0.7 g KH_2PO_4, 1.0 g Na_2HPO_4 in 1 L of water, final pH 7.2.
4. Emersion oil.

7.2.5 Materials for observing exflagellation

1. Normal serum (preferably human serum), pH of 8.0.
2. RPMI medium: 16.4 g RPMI 1640 containing 25 mM HEPES and 2 mM glutamine (keep at –20°C until use).
3. Sodium bicarbonate.
4. PSN: Penicillin 5000 U/mL, streptomycin 5 mg/mL, Neomycin 1 mg/mL.
5. Hypoxanthine.

6. NaOH.
7. Fetal calf serum (FCS).
8. Petroleum jelly: Vaseline®.

7.2.6 Materials for artificial feeding

1. Membrane feeders: Feeders can be of many types but are usually based on the original design of Wade (1975, 1976). (Suppliers include KEMA Nederland B.V., Utrechtsweg 310, 6812 Arnhem. P.O. Box 9035, 6800 ET Arnhem, The Netherlands, and T.P. Young Scientific Glassware, Queens Lane, Bridge of Allen, Stirlingshire, Scotland).
2. Baudruche membranes (Joseph Long, Inc., 69 Roosevelt Avenue, Belleville, New Jersey, 07109, USA) or Parafilm.

7.2.7 Ookinete culture

1. Syringes: 5 and 10 mL.
2. Whatman CF11 other media e.g. Cellselect™ columns (Becton and Dickinson, Denderstraat 24, B9320 Erembodegem-Aalst, Belgium) or Plasmodipure™ filters are more efficient but are much more expensive.
3. Glass wool.
4. Normal mouse serum or FCS.
5. Phosphate buffered saline (PBS).

7.2.8 Materials for monitoring mosquito infections

1. Syringes: 1 mL with 21 G needle.
2. Mercurochrome (Sigma Chemicals).
3. Petroleum jelly: Vaseline®.
4. Glutaraldehyde or formaldehyde: 1% in PBS.
5. Minimum essential medium (MEM): complete liquid medium or powder, made up according to manufacturers' instructions.
6. Teflon glass homogenizer: loose-fitting.
7. Haemocytometer (Neubauer chamber).
8. 0.5 and 1.5 mL microfuge tubes.
9. Glass slides and coverslips.
10. Horizontal centrifuge (with swing-out rotor) capable of spinning at 3000 **g**.

7.2.9 Materials for Sporozoite ELISA

1. Mouse monoclonal antibody (MAB) against circumsporozoite protein (CSP) repeat epitope.
2. Bicarbonate coating buffer: 1.59 g Na_2CO_3 and 2.93 g $NaHCO_3$ in 1 L distilled water, final pH 9.6.
3. Tween 20.

4. PBS.
5. Bovine serum albumin (BSA).
6. Sodium phosphate.
7. Biotin-conjugated anti-CSP antibody.
8. HEPES.
9. Sodium chloride.
10. Avidin alkaline phosphatase (Vector Laboratories).
11. *p*-Nitrophenol-phosphate: 2 mg/mL (Sigma).
12. Bicarbonate developing buffer: 1.58 g Na_2CO_3, 2.93 g $NaHCO_3$ and 200 μL of 4.9M $MgCl_2$ – made up to 1 L in ddH_2O, pH 9.5.

7.2.10 Materials for anaesthesia of rodents

1. Saggatal®.
2. Rompun® (Bayer).
3. Ketamine (Vetelar®) (Parke-Davis).

7.3 METHODS

7.3.1 Parasite passage

For routine parasite passage 10^7–10^8 infected RBC are inoculated by the intraperitoneal (IP) route into a recipient host. Where the parasite has a preference for reticulocytes (e.g. *P. berghei, P. yoelii*) the host may be treated up to 3 days previously with 0.2 mL of a 1.2 mg/mL solution of sterile phenyl hydrazine. The resulting RBC lysis induces a reticulocytosis which permits a higher initial parasite growth rate, giving an earlier and higher gametocytaemia, before infectivity is suppressed. To achieve the same objective some workers simply give a very high initial inoculum e.g. 10^{10} parasites intravenously (IV).

7.3.2 Parasite cryopreservation

Upon each rodent–rodent blood passage chromosomal abnormalities accumulate and the ability to produce gametocytes is rapidly lost (e.g. within as few as eight cycles). It is wise, therefore, to cryopreserve parasitized blood from a host infected by sporozoites.

(a) Freezing infected blood

1. Put 0.1 mL of a solution of 300 U/mL (1 mg/mL) preservative free heparin in a syringe. Using a 21 G needle take blood (preferably containing a high proportion of immature asexual parasites) from an infected host and add dropwise, with shaking, an equal volume of 30% glycerol v/v in PBS (or with DMSO to a final concentration of 10%). Do this in a cold room at 4°C, or on ice.
2. Aliquot blood/cryopreservative mixture into cryotubes or straws on ice, preferably in volumes of less than 0.5 mL.

3. Freeze tubes/straws, ideally using a programmable cell freezer with the following schedule of cooling, and using a 'cold finger' ice-seeding device at step IV (below).
   i. –0.5°C/min to –2°C.
   ii. Hold at –2°C for 15 min.
   iii. –0.5°C/min to –9°C.
   iv. –0.3°C/min to –12°C.
   v. –1°C/min to –25°C.
   vi. –2°C/min to –70°C.
   vii. –5°C/min to –176°C.
   viii. Store at –176°C.

   If no programmable freezer is available: place tubes inside a small thin walled expanded polystyrene box and store in a –80°C freezer (or in solid CO_2) for 24 h. Then transfer vials directly to liquid nitrogen or freezer at –176°C (Note 1).

(b) Thawing

1. Preheat a water bath to 37°C (Note 2).
2. Immediately transfer to ice or subinoculate IP into a recipient host.

7.3.3 Preparation of mosquitoes for blood-feeding

1. Collect adult *An. stephensi* mosquitoes by vacuum aspiration from above pupal bowls directly into feeding cages, or collect pupae by vacuum aspiration into stock vessel.
2. Transfer pupae into open bowls containing fresh clean water (either charcoal filtered tap water, or distilled water containing fresh water aquarium salts) in mosquito feeding cages.
3. Allow pupae to emerge. Pupal collection is the preferred method as the adults are not damaged and therefore feed optimally.
4. For routine parasite transmission, mixed populations of mosquitoes can be fed without difficulty. For experimental infections hungry female adults are selected for feeding vigour. Place a warm container, or the operator's hand on the stock cage, and collect the mosquitoes using a mechanical aspirator.
5. Transfer known numbers of females into feeding containers.
6. Leave the mosquitoes in these containers until at least 3 days post-emergence ideally at 26°C and 70–80% RH, then remove fructose/PABA for between 12 and 24 h before blood-feeding.
7. At least 30 min before feeding take the mosquitoes to the feeding place, e.g. a room or incubator at 20–21°C that is draught and disturbance free, and which can be left under controlled lighting conditions.

7.3.4 Monitoring parasitaemia

(a) Giemsa staining of blood films

1. Make very thin blood films, and air dry as quickly as possible without overheating (a hair dryer is very useful).

2. Fix in absolute methanol for 30 s.
3. Transfer whilst still 'wet' with methanol into 10% Giemsa stain in Giemsa buffer for a minimum of 10 min and up to 45 min in 5% stain. Use the stain only in the first hour following dilution.
4. Following staining wash the slide *very briefly* in running tap water (if not acidic), otherwise rinse briskly in Giemsa buffer. The purpose of the wash is only to remove excess stain; if the slide is left in the buffer the stain will rapidly be washed out of the cells which will then be difficult to examine.
5. Dry in air, standing the slide vertically to dry as quickly as possible (but do not overheat the slide).
6. Parasites are readily observed by oil immersion microscopy at objective magnifications of greater than 40×. It is necessary to observe male and female gametocytes in the blood film (they accumulate on the trailing edge of the film close to the white blood cells). For many experiments it is necessary to do quantitative counts, for this purpose the non-random distribution of the parasites is a critical confounding factor (Notes 3 and 4).

(b) Exflagellation

For many purposes a short-cut to monitor a blood infection for potential mosquito transmission is to monitor the presence of viable male gametocytes in the exflagellation test.

1. Take one small drop of blood, e.g. 1–20 μL, and add immediately to 10 vols of either normal serum (we find human serum best) previously equilibrated with air to a pH of 8.0, or to RPMI medium, 2.0 g sodium bicarbonate, 10 mL PSN, 50 mg hypoxanthine. Make up to 1 L with ddH_2O. Adjust to pH 8.4 with 10 mM NaOH. Sterilize by filtration. Add 10% sterile FCS. Aliquot and store at –20°C.
2. Place a coverslip previously 'edged' with petroleum jelly over the mixed blood/serum, and incubate for 15–20 min at 21–26°C (21°C *P. berghei*, 26°C *P. yoelii* etc.; Table 7.1).
3. Press the coverslip until a *contiguous* monolayer of erythrocytes prevails and observe under dark field-, phase- or interference contrast-microscopy with a 20× or 40× lens.
4. Exflagellation of the microgametocyte is seen as violent centres of movement usually for periods of 30–40 min after addition of the diluent, followed by the escape of individual motile flagella. Intermittent bursts of activity from the long flagella is typical. (Sustained movement could easily be bacterial contamination!). If exflagellation has finished before observation can start, clumps (*not* rouleaux) of red cells indicate where exflagellation has previously occurred.

7.3.5 Blood-feeding of mosquitoes

1. Having prepared the mosquitoes for feeding, the infected rodents are monitored (Sections 7.3.4a and b).
2. If the parasitaemia, gametocytaemia and exflagellation are acceptable, the rodent is anaesthetized by intramuscular inoculation of Rompun/Vetalar into the hind leg (Section 7.3.10). Keep the rodent in a lidded cage until anaesthetic takes effect.

3. When anaesthetized, shave the belly of the rodent with scissors or mechanical trimmers to remove the long and rigid guard hairs.
4. Lay the rodent on the cage and place an impenetrable layer between the cage and the head (biting on the head irritates even a fully anaesthetized mouse). The mosquitoes can be stimulated by breathing into the cage to encourage rapid feeding.
5. The host will remain quiescent for 30–45 min, after which recovery is very rapid.
6. Leave the feeding mosquitoes undisturbed and draught free and in the dark for 30 min. If they need observation use a dark red light.
7. Should the feed not proceed well, check the temperature of the host. Some anaesthetics notably barbiturates cause a noticeable drop in host body temperature. This is deleterious to feeding. In this case a small electric blanket, or a *small* polythene bag filled with warm water (38°C) can be placed over the host. Remember that the anaesthetized host could easily be suffocated by the weight of the water bag (Note 5).
8. After feeding, watch the host recover (in a warm room), then return the fructose to the mosquito cage. Keep the cage at 21°C (e.g. *P. berghei*) or 26°C (e.g. *P. yoelii*; Table 7.1) at 70–80% RH until required.

7.3.6 Removal of unfed mosquitoes

If for experimental purposes it is necessary to remove any unfed mosquitoes, two strategies are available:

1. Withhold the reintroduction of the fructose feed for at least 24 or even 48 h. All males and most unfed females will die – and some fed ones also. This is by far the simplest technique but the dead mosquitoes should be removed as they encourage mould and mite infections.
2. Remove by mechanical aspiration all unfed and partially fed mosquitoes. It is advisable to remove the latter because mosquitoes that have only partially fed rarely become infected and are therefore inappropriate to include in assays of parasite transmissibility.

7.3.7 Membrane feeding

1. Prepare mosquitoes as described above (Section 7.3.3), i.e. aspirate an appropriate number of hungry mosquitoes into the chosen container the night before feeding. In contrast to live host feeds, we find that for membrane feeding the number of mosquitoes is quite critical. If there are too many then they disturb each other, if too few, the proportion that feed is low.
2. Place the membrane (preferably Baudruche) over the feeder and secure tightly. If Parafilm is used, it should be stretched very well in both directions, placed on the feeder, and sealed tightly (Note 6).
3. Switch on the heating to the membrane feeder. The circulating water should be at 38–40°C in most feeder types to ensure the blood in the feeder is at 37–38°C (it is worth testing this accurately for each feeder type – there is wide variation). Heating the blood to above 39°C is deleterious to parasite survival.

4. For gametocyte feeds use a heparinized syringe (Section 7.3.2a) to collect 1 mL of blood from the infected rodent.
5. Keep this blood above 37°C if it is to be 'manipulated' before feeding and you wish to ensure intracellular inactivated gametocytes are to be introduced into the feeder. Otherwise the blood can be stored for up to 30 min on ice (but with progressive loss of infectivity).
6. When the circulating water is at the required temperature, put on the Parafilm membrane and add the blood, ensuring there are no air pockets and that the membrane is not ruptured during introduction of the blood.
7. It is not normally necessary to agitate the blood, though some experienced exponents of infection with cultured *P. falciparum* gametocytes advocate this. If stirred this should be very gentle, e.g. 10–20 rpm and vibration free. Similarly any circulating water pump should also be vibration free - mosquitoes do not feed well if there is a detectable movement on the feeder.
8. Infection is usually only effective in those mosquitoes feeding within 30 min of introduction of the gametocyte infected blood. Conditions for post-feeding manipulation are as given in Section 7.2.7.
9. If mosquitoes are fed on ookinetes or zygotes the blood is also maintained at 37°C (despite the ookinete normally developing at 21–26°C); mosquitoes are not attracted to the feeder otherwise. Haematocrit should be adjusted to 40–50%. In this case feeding can be continued as long as necessary to achieve the required efficiency of feeding, the ookinetes being very long-lived. For this reason alone ookinete feeding is a preferred technique for routine *large scale* parasite transmission.

7.3.8 Maintenance of infected mosquitoes

1. Infected mosquitoes are very delicate for the first 24 h following blood-feeding and are best left undisturbed for this period.
2. Remove unfed mosquitoes as described in Section 7.3.6.
3. Maintain the mosquitoes on 8% fructose/0.05% PABA (or a suitable alternative sugar source) *ad libidum*. Care should be taken to ensure this does not become contaminated with bacteria or yeasts, both of which can result in significant deaths in the mosquitoes and produce extremely fragile guts for dissection (Notes 7 and 8 for critical information regarding temperature and humidity requirements).
4. If possible, the cage should be cleaned of blood droplets because these attract fungi which can reduce mosquito survival.
5. If the cage is sorted in a cold room this is a convenient time to remove the contaminated filter paper from the cages. When conducting long-term infections, i.e. 14–36 days, mite infestations can be a very serious problem if the insectary is not kept scrupulously clean; consequently any dead mosquitoes should be removed from the cages.
6. A second uninfected blood meal on days 4–6 significantly increases mosquito survival, promotes rapid parasite growth and increased sporozoite numbers (Ponnudurai *et al.*, 1989; Ranawaka *et al.*, 1993). Exclusion of an egg bowl also increases nutrients available for parasite growth.

7.3.9 Ookinete culture

1. Take rodents with a suitable high gametocytaemia, where the viability of the microgametocytes has been confirmed by the exflagellation assay. It is not necessary to take infections at the peak of natural infectivity to the mosquito; high parasitaemias can be tolerated because the blood will be heavily diluted and depleted of white blood cells.
2. To remove WBC, prepare for each mL of infected blood 2 mL of Whatman CF11 in a clean syringe barrel (alternatively use Cellselect columns or Plasmodipure filters). Use a glass wool plug to prevent cellulose from eluting from column.
3. Prepare ice cold ookinete medium (Section 7.3.4b). At least 15 vol the amount of infected blood is required. Wash the ookinete medium through the column until the pH of the eluted medium is the same as that applied.
4. Collect blood by sterile technique into a heparinized syringe (Section 7.3.2a). Mix well and store on ice if absolutely necessary (e.g. to pool blood). It is not necessary to use absolute sterile technique – ookinete development occurs well in medium containing penicillin, streptomycin and neomycin.
5. Add neat blood, allow it to adsorb into the column, then wash through rapidly with ookinete medium. Collect *only* the concentrated blood fractions, measure roughly the volume of the fractions collected – make up to 10× the original blood volume with fresh ookinete medium at the desired temperature for exflagellation (e.g. 21 or 26°C).
6. Transfer the parasites to any shallow sterile container suitable for tissue culture, such that medium depth is no greater than 3 mm (or surface area to volume exceeds 3 cm^2/mL). Store in air for 24 h at required temperature (Table 7.1).
7. After 24 h make a blood film (gently incline the container and sample the cells that have settled to the bottom of the culture). Ookinetes are typically concentrated in the tail of the blood film and have a characteristic banana shape, with a condensed spherical nucleus.
8. These cultures can be reconstituted to 50% haematocrit in normal mouse serum or FCS, and used for mosquito infection by membrane feeding (Section 7.3.7, step 9).

7.3.10 Monitoring mosquito infections

It is advisable to monitor mosquito infections even for routine transmission of infections. It is usual to monitor oocyst infections about halfway through their development, e.g. 10–12 days. However it is sometimes necessary to monitor each and every stage of development. Appropriate methods for enumeration of infections are discussed in the Introduction.

(a) Observing gametogenesis

1. Mosquitoes should be collected within 10 min of feeding and preferably within 1 or 2 min of engorgement.
2. Knock down mosquitoes with CO_2 (ice inhibits gametogenesis and stunning smashes the mosquito on the walls of the container!).
3. The simplest method is to puncture the insect thorax with a needle and proceed to step 4 below. Alternatively, rapidly remove the head of the mosquito (and, if

you need to, the wings and legs – though this is not necessary for the skilled dissector). Make a small incision in the cuticle of the penultimate segment of the abdomen using a sharp blade (a 21 G needle on its side is excellent, although some workers prefer a Shute needle). Use a simple dissecting microscope for this.

4. With one needle holding the thorax *gently* pull directly backward on the last segment of the abdomen – the blood meal will gradually be extruded. Take your time to allow the congealing blood to 'flow' out with the midgut.
5. Transfer blood meal into a small drop (e.g. 2 μL) of ookinete medium.
6. Cut through the midgut epithelium (if not already ruptured) and gently irrigate the midgut in the medium. Remove the midgut tissue and mix the blood and medium. Either make a blood film (if dense) or with a circular motion make an expanded droplet.
7. Dry preparation quickly using a hair dryer or a hot-block at 37°C. Stain with Giemsa stain or observe exflagellation directly by phase contrast or interference microscopy (Section 7.3.4b). Microgametes have thin elegantly sinusoidal flagella 15–20 μm long, usually single, and often without nuclei. They can be confused with fibrin strands in Giemsa stained preparations where clotting has occurred; these are however rarely of sinuous contour.

(b) Observing ookinetes

1. Mosquitoes should be collected at 24 h (or earlier if retort-form parasites (9 h) or zygotes (<5 h) are required).
2. Dissect and prepare blood films and stain in Giemsa stain as in Section 7.3.10a. An alternative strategy to look specifically for mature ookinetes is to take the midgut and suspend it in calcium-free medium, e.g. PBS, to dissociate the midgut cells. The tissue (now comprising only the basal lamina of the gut) is gently rinsed clear of the blood meal and midgut cells. If this tissue is now dried onto a slide it will contain a selected population of ookinetes that have successfully crossed the epithelium and attached to the lamina prior to becoming oocysts (Simonetti *et al.*, 1993).
3. Zygotes are, to the inexperienced eye, indistinguishable morphologically from macrogametes and macrogametocytes. Retort-form ookinetes, as their name suggests, look like the alchemist's glass retort, where the 'spout' is the emerging apical complex. The 'body' of the retort becomes progressively smaller as the ookinete matures. Parasites with very thin prominences are typical of degenerating/abnormal cells (in well prepared, rapidly dried specimens). Mature ookinetes are banana shaped, with a central magenta coloured spherical nucleus, the 'stalk' of the banana is the apical prominence of the ookinete where the rhoptry/micronemes secrete their content to the exterior.

(c) Observing oocysts

1. It is preferable not to use mosquitoes with a retained blood meal when dissecting for oocysts. Mosquitoes are usually dissected around day 10 for oocysts; earlier and the inexperienced operator might confuse midgut nuclei with parasites, later many oocysts may have burst.
2. Dissect the midgut as in Section 7.3.10a, and remove the Malpighian tubules, hindgut, crop and foregut (if attached). Five midguts can easily be prepared on a single microscope slide.

3. For immediate examination simply place a Vaseline® rimmed coverslip on the guts and withdraw fluid until the guts change from white to translucent grey colour. Observe quickly by phase contrast or interference microscopy (Note 9).
4. Oocysts are almost spherical objects of between 4 and 60 μm diameter (depending on age). Their 'edge' is usually sharply defined and a string of dark pigment granules is visible in the cytoplasm. Do not confuse with epithelial cell nuclei, with host cell vacuoles, air bubbles or detached midgut cells.
5. From day 10 onwards sporozoites can be seen with increasing frequency budding from the oocyst cytoplasm in various patterns. Dying oocysts are highly vacuolated or may, on rare occasions, be brown coloured (melanized).
6. Oocysts can readily be stained with 0.1% mercurochrome in PBS for between 5 min and 1 h, and can be observed by bright-field microscopy in the staining solution. Oocysts stain pink to brick red against a pale red midgut background. Permanent preparations can be made by transferring the stained guts into 1% glutaraldehyde or formaldehyde in PBS.

(d) Observing sporozoites

Sporozoites may be obtained from mature oocysts by crushing/homogenizing midguts from day 12 of infection onwards. These are immature sporozoites capable of invading the salivary gland but incapable (less capable) of infecting vertebrate hosts or hepatocytes. Alternatively, mature sporozoites infectious to vertebrates can be obtained from salivary glands, or whole body extracts. There is little value in using whole body extracts as the population of sporozoites is mixed and not adequately defined for important biological and biochemical properties; the method is included here only for completeness.

(i) Midgut sporozoites

1. Midgut sporozoites can be obtained by dissecting midguts bearing mature oocysts (e.g. days 15—20) as in Section 7.3.10a into MEM medium.
2. Transfer to a *loose*-fitting Teflon glass homogenizer (prechecked to ensure it does not homogenize the sporozoites) on ice.
3. Homogenize gently (e.g. three gentle vertical movements without rotation), leave plunger in and remove supernatant.
4. Count sporozoites in a haemocytometer. Count all 16 cells in each of the four corners of the Improved Neubauer chamber. Calculate the mean number in 16 cells, multiply by 10^4, this is the number of sporozoites/mL.

(ii) Total sporozoites (Ozaki *et al.*, 1984)

1. Puncture a 0.5 mL microfuge tube with a 21 G needle.
2. Insert a glass wool plug. Place inside a 1.5 mL microfuge tube from which the lid has been removed – ensure the lip of the 0.5 mL tube rests securely on that of the 1.5 mL tube.
3. Remove wings, head and legs from the anaesthetized mosquitoes and cut the abdomen from the thorax, place either or both into 200 μL MEM medium in the smaller tube.
4. Centrifuge in a horizontal (or swing-out rotor) at 3000 g for 2–3 min.

5. Add 100 μL of fresh medium to the top tube and spin again. Collect sporozoites from the bottom tube.

(iii) Salivary gland sporozoites

Salivary glands must be dissected to ensure the sporozoites are from these organelles and are not in transit in the haemocoele of the thorax. Experts pull heads from anaesthetized mosquitoes dragging with them the salivary glands from the thorax; this is a very clean dissection but slow. Alternatively one can cut the head off and express the glands from the thorax; this is a faster technique but the final preparation is always contaminated with mosquito muscle cells and other debris. The latter will be described here. Other techniques are elegantly described by Shute and Maryon (1966) and Vanderberg and Gwadz (1980).

1. Anaesthetize mosquitoes with CO_2 and place individually or in groups on a microscope slide (a plastic slide is useful as it is hydrophobic). Cut all the heads off *quickly* to avoid recovery of mosquitoes.
2. Place a drop of medium on slide. Bring cut neck of mosquito close to the drop of medium and gently press down on the thorax using the inclined face of the syringe needle (do not push forward as this will puncture the thorax and prevent the internal pressure from expelling the haemocoelomic fluid).
3. Using the sharp edge of the other needle cut the very first drop of tissue to be expelled from the severed neck. This contains the salivary glands and some other tissues.
4. The glands are two three-lobed opalescent structures in the droplet. Gently tease from the other tissues and transfer to a clean area of the drop or to a homogenizer tube as above (Section 7.3.10d(i)).
5. If simple observation of the glands is needed place a coverslip on the drop and tap firmly on the coverslip to burst the glands beneath. Sporozoites can be seen as 'snake-like' cells 10–20 μm long and 1 μm in diameter flooding from the ruptured glands.

(iv) Sporozoite ELISA assay

Despite its drawbacks many people use the anticircumsporozoite ELISA test to measure mosquito infections (Wirtz *et al.*, 1985).

1. Coat ELISA plates with 10 μL purified monoclonal antibody directed against the CSP repeat epitope, at 10 μg/mL in bicarbonate coating buffer. Incubate at 4°C for 24 h.
2. Wash wells 3× with 100 μL 0.05% Tween 20 in PBS (PBS-T).
3. Block non-specific binding sites with 100 μL 0.05% bovine serum albumin in PBS at 37°C for 30 min.
4. Wash wells 3× with 100 μL PBS-T.
5. To reference wells add 100 μL of appropriately diluted samples of known sporozoite controls in 0.05% BSA, 0.5% NP4O in PBS. Add buffer alone to background control wells, and add 100 μL of unknown sporozoite suspensions (also prepared in 0.05% BSA, 0.5% NP4O in PBS) to test wells. Uninfected mosquito preparations are good negative controls. Incubate at 37°C for 2 h.
6. Wash wells 3× with 100 μL PBS-T.

7. Add 100 μL of biotin conjugated anti CSP antibody (the repetitive nature of the CSP epitope recognized by the monoclonal antibody, permits use of the same antibody for both adsorption and assay) diluted e.g. 1:500 in PBS-T. Incubate 1 h at 20–25°C.
8. Wash as previously.
9. Add 100 μL avidin-alkaline phosphatase diluted 1:400 in 10 mM HEPES, 0.15 M NaCl and 0.1% Tween-20. Incubate 30 min at 37°C.
10. Wash 5× in PBS-T.
11. Incubate with 100 μL *p*-nitrophenol-phosphate (2 mg/mL) in bicarbonate developing buffer, for an 'appropriate' period.
12. Read optical density at A_{405}.
13. Subtract background from all values and then subtract $A_{\text{uninfected mosquito control}}$ from $A_{\text{infected mosquitoes}}$. For the standardization curve plot the OD against known sporozoite number.
14. Compare ($A_{\text{experimental}} - A_{\text{uninfected mosquito control}}$) with standard controls to determine sporozoite 'equivalent' number.

7.3.11 Anaesthesia of rodents

(a) Routine blood-feeding for mosquito maintenance

For routine blood-feeding of stock cages, large rodents and rabbits can be anaesthetized with Saggatal® at a rate of 0.125 mL/100 g body weight. The disadvantages with this anaesthetic are that hosts become less sensitive upon repeated use and that the body temperature of the host is reduced making it less attractive to the mosquito. For these reasons it is not advised for experimental work.

(b) Experimental anaesthesia

Extensive work at the University of Nijmegen in the 1980s led to adoption of a mixture of ketamine and the muscle relaxant Rompun as a preferred anaesthetic for small and sensitive rodents.

1. Mix 1 vol of a stock 2% solution of Rompun® with 2 vol of Ketamine (Vetelar®). For small animals this should be diluted to half-strength by addition of 3 vol of sterile PBS. Both these solutions can be stored at 4°C until required.
2. The dose rate is 0.025 mL of the original stock mixture (i.e. 0.05 ml diluted mixture) per 10 g body weight. It is given by the intramuscular route in the rear leg musculature. Some bleeding may occur at the local site of inoculation.

7.3.12 Parasites used

A full list of rodent malarial parasite species described up to 1978 is found in the classic text Rodent Malaria (Killick-Kendrick, 1978). They included *P. berghei, P. yoelii, P. vinckei and P. chabaudi* and were summarized in Table II of the above reference, which is reproduced here as Table 7.2.

Numerous clones and isolates of each of these species and some of the subspecies have since been prepared notably at the University of Edinburgh, and at the Prince

Table 7.2 Geographical distribution of species and subspecies of African murine malaria parasites. Modified from Killick-Kendrick (1978)

	Groups of parasites			
Places	P.berghei *group*	P.vinckei *spp.*	P.chabaudi *spp.*	*Other*
Katanga	*P.berghei* *P.yoelii* sp.(?)	*P.v.vinckei*		
CAR	*P.y.yoelii*	*P.v.petteri*	*P.c.chabaudi partim*	
Brazzaville	*P.y.killicki*	*P.v.lentum*	*P.c.adami*	
Nigeria	*P.y.nigeriensis*	*P.v.brucechwatti*		
Cameroons	*P.yoelii* ssp.	*P.vinckei* ssp.		
Kenya	*P.yoelii* ssp.			
Egypt				*P.aegyptensis* A

Leopold Institute of Tropical Medicine, Antwerp. Researchers are directed to these institutes for a comprehensive list of clones and their various properties. Many are defined for their resistance/susceptibility to the major antimalarial drugs, their ability to produce gametocytes and for some of the antigens expressed. The list is too long to be quoted here, further details are contained in: Beale *et al.* (1978); Janse *et al.* (1991); Killick-Kendrick (1978); Lainson (1983); Montalvo-Alvarez *et al.* (1991); Said *et al.* (1986) and Walliker (1983).

NOTES

1. Use appropriate safety procedures in handling liquid nitrogen.
2. Liquid nitrogen may have entered the cryotube. There is therefore a significant risk of explosion immediately the tube is removed from the liquid nitrogen. You are advised to place the tube immediately in a protective metal container whilst you *loosen the cap*. Put tube in a warm water bath until blood is fully thawed (1–3 min).
3. We have found that two simple transects in a 'St. Andrew's Cross' on the blood film closely approximates to the proportional count of an entire blood film (Young and Sinden, unpublished). 10 000 RBC should be screened for an 'accurate' gametocyte count, but only 1000 RBC need be counted for an accurate estimate of asexual parasite distribution (rings, trophozoites and schizonts). Russell *et al.* (1963) suggest that to obtain parasite counts that do not exceed 10% error the following formula should be used:

   $$N = 45.5[(I-P)/P]$$

   where N is the number of RBC to be counted, P is the number of parasites per unit number of red cells, and I is the selected unit (i.e. 10 000 RBC).
4. Young gametocytes of both sexes are easily confused both with each other and with asexual trophozoites (i.e. for the first 18 h of the 24–26 h growth cycle) and are often scored as macrogametocytes. It should be acknowledged therefore that macrogametocytes are usually overestimated. The only reliable count is on the mature microgametocyte which has a distinct cytoplasmic coloration (from pale grey to brick red depending upon staining conditions). Vacuolated gametocytes occur in 'mature' infections and may be nonviable.
5. Each mosquito takes on average 1.5 μL of blood. The use of large numbers of mosquitoes can be lethal to small rodents, e.g. a mouse should not lose more than 1–1.5 mL blood if recovery is to be allowed.
6. Whilst Baudruche membranes can be prepared in advance, attach Parafilm membranes only just before adding the blood; Parafilm will 'dry-out' and become very weak. Neither should an empty feeder be placed against a cage whilst hot – the Parafilm is quite sticky if heated above 37°C and sticks to the cage and is then easily torn if the feeder is jogged. Baudruche membrane has no such problems.
7. Temperature control is critical particularly in the first few hours of infection when gametogenesis should occur (Table 7.1). At later stages of sporogony both the absolute temperature and the duration of exposure to elevated temperatures are important; high temperatures (e.g. 30°C) kill the parasite quickly, lower temperatures (24–28°C, depending on species) will be lethal but only after prolonged

exposure (days). Reduced temperatures e.g. 10–18°C, although not lethal, slow down parasite development very markedly and are not advised unless you wish to sustain an established salivary gland infection. The practice of using low temperatures (i.e. 0–4°C) to knock-down a cage for examination is not advised in the first 5–12 h of infection – this can kill the parasite probably indirectly by inhibiting exflagellation or early sporogonic development. Established oocyst infections, although not killed, can be significantly slowed by such treatments. Mosquitoes required for examination should ideally be removed by aspiration (see below).

8. Humidity should be maintained at 70–80% at all times. Lower humidity results in progressive desiccation of the insect, higher humidities promote fungal growth and loss of mosquitoes stuck to wet surfaces.
9. It may be necessary to change focus to observe both top and bottom sides of the gut. If you don't use the Vaseline rim the pressure exerted on the gut as the medium evaporates will burst the gut.

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CHAPTER EIGHT

Infection of mosquitoes with primate malaria

William E. Collins

8.1 INTRODUCTION

The primate malarias are those species of *Plasmodium* infectious to humans, apes, New World and Old World monkeys and lemurs. The four human-infecting parasites, *P. falciparum, P. vivax, P. malariae* and *P. ovale* are also infectious to New World monkeys and chimpanzees. The most studied monkey-infecting species, *P. knowlesi, P. cynomolgi, P. gonderi, P. coatneyi, P. fragile, P. fieldi, P. simiovale, P. simium, P. inui,* and *P. brasilianum* are infectious to either New World or Old World monkeys or both. New species and strains of primate malaria parasites are periodically isolated and described. The materials and methods presented here are those used under controlled conditions for the production of infected mosquitoes, parasites for infection and transmission studies, and the use of sporozoites from *Plasmodium*-infected mosquitoes to infect other hosts and cultures.

The requirements for such studies are mosquitoes capable of supporting the development of a particular species or strain of parasite of interest and a primate host, usually a monkey, but in some instances a chimpanzee. Requirements include, therefore, an insectary to support and contain exotic species of *Anopheles* which may be infected with human pathogens. An approved animal facility staffed by qualified veterinarians unless all work is to be done with naturally infected humans. In this case, containment facilities for infected mosquitoes are vitally important.

Few species of primate malaria have been adapted to continuous *in vitro* culture. One species which is routinely grown is *P. falciparum*. Some strains of this parasite readily produce gametocytes in culture which can serve as a source for mosquito infection. Although other species such a *P. cynomolgi, P. fragile, and P. inui,* have been so adapted, mosquito infection has been obtained rarely. Brief methods will be presented on the culture of parasites for mosquito infection and the production of exoerythrocytic stages by the introduction of sporozoites to primary hepatocyte cultures.

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8.2 MATERIALS

8.2.1 Materials used in mosquito infection

1. Karo syrup solution: Karo syrup is a commercially available corn syrup (CPC Food Service, Englewood Cliff, NJ). A 10% (v/v) solution is prepared in dH_2O. If not available, a 10–20% sucrose/fructose solution can be substituted.
2. Mercurochrome solution: 2% merbromin, commercially available from pharmacy retailers. Can be used full strength or diluted to 1% working concentration in PBS.
3. Vacutainer tubes: Sodium heparin or Acid/Citrate/Dextrose (ACD), commercially available (Becton-Dickinson, Rutherford, NJ).
4. Phosphate buffered saline (PBS): 0.01 M, pH 7.2. Can be made up in dH_2O and autoclaved for sterilization.
5. 20% monkey serum/saline: v/v solution of heat-inactivated serum in PBS.

8.2.2 Materials for use with primates

1. Ketaset: Ketamine hydrochloride (Fort Dodge Laboratories, Inc., Fort Dodge, Iowa).

8.2.3 Giemsa staining of blood films

All reagents used for making Giemsa stain should be free from water; only freshly opened bottles of methanol and glycerine should be used. The brown bottle and glass beads should be clean and completely dry.

1. Giemsa stain (stock): Add 3 g of Giemsa (Fisher, Certified Biological Stain), 270 mL of absolute methyl alcohol (acetone free), and 140 mL of glycerine to a clean, dry 500 mL brown bottle which contains 15 mL of glass beads (size 3 mm). Shake each day for at least 30 min to 1 h for 2 weeks. The quality will improve with age. Be sure to keep tightly closed. Before use, filter an aliquot of stain through a Whatman #1 filter paper into a small dry brown glass bottle. Keep tightly capped. Never use a wet or dirty pipette in the Giemsa. Be sure to shake the bottle before filtering.
2. 100× stock buffer solution: Add 130 g of Na_2HPO_4, 70 g NaH_2PO_4 ($NaH_2PO_4 \cdot H_2O$), and 400 mg of sodium azide (0.02%) to dH_2O, mix, and adjust the final volume to 2000 mL. Store in brown bottles until use. The 1× stock buffer is prepared by diluting the concentrated buffer in dH_2O. The final pH should be 7.0.
3. Triton stock solution: Add 15 mL of Triton X-100 and 60 mg of sodium azide into 285 mL dH_2O, mix, and shake gently at 37°C until dissolved.
4. Giemsa stain solution: Mix 1 mL of filtered Giemsa stock with 50 mL of 1× staining buffer and two drops of Triton solution. Mix well but avoid bubbles.

8.2.4 Materials for freezing and thawing blood and parasites

1. Glycerolyte 57 solution: The glycerolyte freezing solution can be obtained commercially (Fenwal Laboratories, Deerfield, IL, USA) or prepared by mixing 57 g

glycerol, 1.6 g sodium lactate, and 30 mg KCl, 124.2 mg Na_2HPO_4, 51.7 mg NaH_2PO_4 ($NaH_2PO_4 \cdot H_2O$), into 100 mL of dH_2O (approximately 6.8 pH). This solution can be sterilized by autoclaving at 15 psi for 15 min.

2. NaCl solutions: Two solutions of NaCl are required, 12% and 1.6%, and one solution of 0.9% NaCl and 2% dextrose. All of these solutions are w/v solutions in dH_2O and can be sterilized either by autoclaving or by filtration through a 0.2 μm pore filter.
3. 50% fetal bovine serum/saline: v/v solution of heat-inactivated serum in PBS.

8.3 METHODS

8.3.1 Methods associated with mosquitoes

(a) Infection of mosquitoes with plasmodia

1. Female mosquitoes (3–7 days after emergence) are transferred to pint ice-cream carton cages 1 day before being fed on infected animals. Up to 150 mosquitoes can be accommodated in each cage. To select female mosquitoes for animal feeding, a warm hand is placed on the side of the carton. Females congregate on the side and are aspirated into pint ice-cream carton cages for feeding on the monkeys.
2. Depending on the species of *Anopheles*, they receive either sugar water, water alone or no moisture in order to 'starve' them prior to feeding.
3. Monkeys are tranquillized and immobilized on 'restraining boards' using Velcro straps. The centre of the restraining board is 'cut out' to allow the belly of the monkey to rest directly on the top of the 2 pint feeding cages.
4. Mosquitoes are allowed to feed to engorgement (usually 8–10 min). Unfed mosquitoes are removed and the fed mosquitoes placed in a large holding incubator (25°C; 60% RH).
5. Mosquitoes are fed 10% Karo solution on a cellulose or cotton sponge daily.
6. Groups of mosquitoes are dissected 7–10 days after feeding (depending on the species of *Plasmodium*), and the midguts examined for the presence of oocysts.
7. Dissected midgut is placed in a drop of mercurochrome which highlights the oocysts and makes accurate counting much easier.
8. Oocysts are counted and recorded.
9. Oocyst counts are recorded either as oocysts per individual gut, mean number of oocysts per gut, or oocysts per positive gut. A Gut Infection Index (GII) is the number of oocysts per 100/guts.
10. Infected lots of mosquitoes are returned to the incubator and maintained until sporozoites are present in the salivary glands (10–18 days depending on the species of *Plasmodium*).
11. Oocysts begin to mature or differentiate 1 or 2 days prior to rupture. Some oocysts may chitinize early in their development and others may degenerate and begin to shrink prior to differentiation. Spores of Ross commonly develop, which indicates a poor parasite/vector relationship. In other instances, oocysts mature to the presence of sporozoites, but the oocysts fail to rupture. It is essential to follow the daily development of oocysts when a new vector/parasite combination is being studied. In some instances, sporozoites are rarely present in the salivary glands even though many oocysts have ruptured.

12. Pre- or post-feeding dissection of the mosquitoes and examination of the salivary glands indicates the number of infected mosquitoes which fed upon the animal being infected. The remaining number of sporozoites in the salivary glands are graded 1+ (1–10 sporozoites, 2+ (11–100 sporozoites), 3+ (101–1000 sporozoites), or 4+ (greater than 1000 sporozoites). The total number of +'s is recorded for all mosquitoes fed on the animal to determine the exposure index for the monkey. The Positive Gland Index (PGI) is the number of +'s per positive mosquito and indicates the efficiency of a particular vector/parasite combination in concentrating sporozoites in the salivary glands.

(b) Infection of mosquitoes through membranes

1. Mosquitoes are prepared for feeding as described above.
2. Blood is collected from the donor *via* venapuncture using heparin or ACD as anticoagulant. EDTA reduces the infectivity of the gametocytes.
3. Blood is fed either undiluted or diluted using a warm-water (37–40°C) circulating bell (Rutledge *et al.*, 1964) covered with stretched Parafilm.
4. The feeding bell rests gently on the top of the netting on the cage.
5. Most species will engorge within 5–8 minutes. Other species, such as *An. dirus* do not feed readily through the membrane and engorgement >50% is rarely obtained even when the feeding time is extended to 20 min.
6. Blood from humans and chimpanzees has remained highly infectious for periods up to 4 h, whereas gametocytes from *P. falciparum* cultures often lose infectivity at a faster rate.
7. Gametocyte infectivity is often markedly inhibited by serum factors present in blood from humans and, particularly, monkeys.
8. In most instances, infectivity is greatly enhanced if the plasma is replaced with blood or plasma from an uninfected host.

8.3.2 Methods associated with primates

(a) Care and management of the primates

1. Upon arrival at the testing facility, an animal history form is completed for each animal.
2. All animals are weighed, TB tested, examined for concurrent intestinal parasitic infections, and when indicated, treated appropriately.
3. Each animal is screened by blood smear and indirect fluorescent antibody (IFA) to identify the presence of previous malarial infections.
4. All animals are treated for medical problems as they arise by the attending veterinarian.
5. All observations, plus parasitologic and laboratory results are recorded on a daily basis and entered into a computerized database.
6. Housing, feeding, and handling of animals: Monkeys are housed singly or in pairs to avoid injuries due to fighting.
7. Space recommendations for laboratory animals should conform to national standards set forth in, for example, the *Guide for the Care and Use of Laboratory Animals*, NIH.

8. All monkeys are fed a diet that has been shown to provide adequate nutrition and calories in captive macaques or New World monkeys such as *Aotus* spp. and *Saimiri* spp. used in malaria-related research.
9. Because of intercurrent illness, some monkeys may require special feeding as designated by an attending veterinarian.
10. Feed must be clean, free of contaminants, and freshly prepared.
11. Animals are handled as little as possible.
12. Monkeys are administered 0.1 mL Ketaset intramuscularly for anaesthesia when they are to be given a physical examination, have blood drawn, be fed upon by mosquitoes and/or be weighed.
13. On days when monkeys are to have blood drawn or to be weighed only, anaesthesia need not be given.

(b) Establishment of baseline values

It is important to constantly monitor the well-being of the experimental primates.

1. Animals are examined twice daily for attitude and general well-being.
2. Any animal showing signs of distress is examined by the animal health technician and treatment, if needed, is given under the supervision of the resident veterinarian.
3. Surgical and treatment facilities should be readily available; additional veterinary assistance should be available, on call, 7 days a week.
4. Animals should be housed in a limited access 'secure' facility.
5. During the preinfection conditioning period, blood collections are made to determine haematological values of each animal.
6. Animals should receive weekly supplements of iron from the beginning of blood collections and/or plasmodial infection to the termination of the infection.

(c) Infection via *inoculation of sporozoites*

1. Infected mosquitoes are killed by exposure to chloroform or chilling.
2. Legs and wings are removed and the bodies transferred to a clean slide (wiped with alcohol just prior to use).
3. Salivary glands are either: (i) after decapitation teased from the neck opening with a minutin needle, or (ii) pulled from the thorax by gently pulling the head with a 25 G needle on a disposable syringe; once the glands are freed, the salivary duct is severed by the edge of the needle.
4. Glands are transferred to a drop of 20% heat-treated monkey serum/saline (homologous species).
5. Salivary glands are then crushed to free the sporozoites. If numbers are small, the salivary glands are crushed under a coverslip. The coverslip is gently removed and the sporozoites 'washed' from the coverslip onto the slide using several drops of monkey serum/saline.
6. Fluid is then aspirated into a syringe using a 25 G needle.
7. Suspension is transferred to a chilled cryovial and the volume increased to approximately 1 mL.
8. Repeated expiration and expression from the syringe is made to further dissociate the sporozoites and salivary gland tissue.
9. A small volume is transferred to a Neubauer cell counting chamber and allowed to set for 10 min. The number of sporozoites per mL is calculated.

10. Sporozoite suspension is diluted to the desired number of sporozoites in 1 mL of serum/saline.
11. Sporozoite suspension is injected into the femoral vein using 25 G or 23 G needles.

(d) Infection with 'fresh' parasitized blood

In addition to infection *via* inoculation with sporozoites, monkeys can also be infected by direct transfer of infected blood between animals.

1. Blood is collected from a donor animal into a heparinized tube.
2. Based on the parasite count, the blood is then aseptically diluted in phosphate-buffered saline (pH 7.0) to give the required number of parasites to be inoculated in 1 mL or less.
3. Recipient animal is restrained and the blood slowly injected into the femoral vein using a 25 G needle.
4. Pressure is maintained on the needle exit hole until bleeding stops.
5. For infection *via* inoculation of 'frozen' parasitized blood, vials of frozen blood are thawed rapidly in a 37°C water bath (described below).
6. Recipient animal is restrained and the blood slowly injected into the femoral vein using a 25 G needle.
7. Pressure is maintained on the needle exit hole until bleeding stops.

(e) Monitoring the infection in the primate

Parasite counts are made by the examination of blood films stained with Giemsa stain.

1. New World monkeys are removed from cages and immobilized by animal handler.
2. Thoroughly clean the back of the leg with an alcohol sponge (followed by a dry wipe).
3. A prick is made on the back of the leg with a lancet, carefully avoiding the vein.
4. From the drop of blood which is expressed, thick and thin blood films are made.
5. For exact quantitation, the technique of Earle and Perez (1932) is recommended.
6. Using a 5 uL pipette, blood is collected and then expressed onto a prescribed area (15 mm × 5 mm) on a precleaned, prelabelled (double-frosted) microscope slide.
7. A second drop of blood is added to the same slide and a thin film prepared.
8. Slide is labelled with the animal identification number and the date.
9. Old World monkeys are anaesthetized before being removed from the cage.
10. After thorough cleaning of the area, a prick is made in the ear with a lancet.
11. From the drop of blood which is expressed, thick and thin blood films are made as above.

(f) Staining of blood films

1. Slides are air-dried.
2. Thin films are fixed by dipping in methyl alcohol and dried in a 37°C convection oven for approximately 5 min.
3. To Coplin jars are added the Giemsa stain solution as described in the Materials section.
4. Slides are immersed in the stain for 45 min.

5. Thin film portions of the slide are dipped in the 1× Giemsa stock buffer solution to remove excess stain.
6. Thick films are immersed in 1× Giemsa stock buffer solution for 3–5 min to destain.
7. Slides are put on racks in the convection oven until dry.

(g) Examination of blood films

1. Using the method of Earle and Perez (1932) (with a microscopic eyepiece containing a Howard disc), the number of parasites per μL of blood is determined by counting the number of parasites in a band through the width of the rectangular blood film.
2. When parasite counts are greater than 2% of the RBCs infected, the number of parasites per 1000 RBC is determined using the thin film. Based on an estimated mean RBC count of 4 000 000 per μL, the number of parasites per μL is estimated (percent infected × 40 000 = parasites per μL).
3. Gametocyte counts for species other than *P. falciparum* or *P. reichenowi* are made on the thin film and recorded per 100 WBC.
4. Whenever possible, separate counts are recorded for the micro- and macrogametocytes.

(h) Collection of parasitized blood

1. Blood for freezing or membrane feeding to mosquitoes is collected from a restrained (New World) or anaesthetized (Old World) monkey *via* the femoral vein.
2. No more than 10% of the estimated total blood volume is removed (estimated volume of 40 mL per kg) except when the animal is transfused with blood from other similar karyotype animals.
3. With New World monkeys, blood is collected using a syringe equipped with a 25 G needle.
4. For larger animals (Old World monkeys), 23 G needles or vacutainers are used.
5. Blood is transferred to serum separation tubes or tubes containing anticoagulant.
6. Pressure is maintained on the needle exit hole until all bleeding stops. If the femoral artery is used, pressure is maintained for at least 5 min and animal is watched after being returned to the cage to assure that all bleeding has ceased.

(i) Processing of blood for freezing

1. Blood is collected and transferred to a heparinized tube.
2. Spin tube for 10 min at 1200 rpm.
3. Estimate RBC packed cell volume (PCV), remove plasma.
4. Calculate 2× RBC PCV. This is the total glycerolyte volume used.
5. Add 1/5 of the glycerolyte volume to cells, using a syringe with a 25 G needle at 1–2 drops/s with shaking.
6. Let stand 5 min.
7. Add remaining glycerolyte at same rate with shaking.
8. Aliquot 0.5 mL to 1 mL per labelled cryotube.
9. Transfer to vapour phase in liquid N_2 freezer.

(j) Thawing of frozen stored parasitized blood

1. Cells are thawed at 37°C and transferred to a 50 mL tube by sterile pipette, volume noted.
2. Poke hole in cap of 50 mL tube with 16 G needle.
3. Take up 0.2× blood volume of 12% NaCl with a small syringe and 21 G needle.
4. Add the 12% NaCl dropwise through hole while shaking tube.
5. Stand at RT for 5 min without shaking.
6. Take a larger syringe and 19 G needle, take up 10× volume of 1.6% NaCl.
7. Add the 1.6% NaCl dropwise through the hole while shaking.
8. Spin at 1400 rpm for 10 min.
9. Remove supernatant by aspiration, suspend pellet by gentle shaking.
10. Add 10× blood volume of 0.9% NaCl and 2% dextrose solution the same way as above.
11. Spin at 1400 rpm for 10 min.
12. Remove supernatant by aspiration.
13. Suspend pellet by gentle shaking, add 1 mL of physiological saline.
14. Inoculate recipient monkey *via* the femoral vein using a syringe and a 25 G or 23 G needle (New *vs* Old World monkey).

(k) Frozen storage of sporozoites

1. Sporozoites are suspended in 50% heat-treated fetal calf serum/saline.
2. Sporozoite suspension stored in plastic cryovials over liquid N_2.
3. Prior to injection, rapidly thaw.
4. Inject directly into the femoral vein of the primate host.

(l) Antimalarial drug treatment

1. New World monkeys of 1 kg or less, infected with chloroquine sensitive parasites by the inoculation of parasitized blood, are treated with chloroquine (30 mg base over 3 days = 15 mg on days 1 and 3 or 10 mg daily on days 1, 2, and 3) given by oral intubation.
2. Similar monkeys infected by sporozoites (either through inoculation or by mosquito feeding) are treated additionally with primaquine (2.5 mg base daily for seven consecutive days).
3. Monkeys infected with chloroquine-resistant strains are treated with a single dose of 20 mg mefloquine given by oral intubation.
4. If drugs are in tablet form, they are ground with a mortar and pestle in 1 mL of water and Karo syrup (1:1), drawn into a syringe and introduced with a 4-inch blunt-ending metal feeding tube.
5. Macaque monkeys 4 kg or greater, infected with nonhuman primate malaria parasites are treated with 300 mg chloroquine (150 mg base on day 1 and 150 mg base on day 3) given by oral intubation.
6. If the infection is sporozoite-induced, animals are also treated with primaquine (7.5 mg base daily × 7 days).
7. Chimpanzees are treated with the same dosages of drug recommended for humans.
8. Injectable chloroquine is usually given because the taste of the drug is objectionable to the animal.

9. Primaquine tablets can be inserted into a banana daily for the 14-day recommended treatment period.

NOTES

1. Most species of *Anopheles*, such as *An. freeborni*, *An. stephensi*, *An. gambiae*, *An. quadrimaculatus* and *An. albimanus* are allowed to mate in the emergence cages. Other species such as *An. dirus*, *An. maculatus*, *An. albitarsis*, and *An. pseudopunctipennis* are force-mated. To force-mate mosquitoes, the male is impaled on a minutin pin attached to a stirring stick. The female is etherized. The head and legs of the male are removed and the claspers brought at a 30 degree angle to the terminalia of the female. Once copulation commences, the male/female are transferred to holding cages. With practice two people, one person to catch males and the other to mate the mosquitoes, 100 mosquitoes can be mated in 30–45 min.
2. Splenectomy of the primate is vital for the induction of high-density gametocytemia in many New World monkeys infected with human-infecting plasmodia. It also enhances gametocyte infectivity in nonhuman primates infected with their natural parasites. All surgeries should be performed by qualified veterinarians under aseptic conditions, and supportive care must be readily available.
3. If exoerythrocytic (EE) stages of malaria parasites are desired, they can be obtained *via* liver biopsies performed on animals following sporozoite inoculation. In some instances, biopsies are made of uninfected animals to obtain hepatocytes for *in vitro* development of EE-parasites depending on the availability of sporozoites. As with splenectomy, this procedure should be carried out by qualified veterinarians in approved facilities.

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CHAPTER NINE

Infection of mosquitoes with filaria

Harold Townson

9.1 INTRODUCTION

The most widespread and common human filarial parasite is *Wuchereria bancrofti*, which currently infects around 100 million people in the humid tropics. The most common vector in towns is the ubiquitous tropical house mosquito, *Culex quinquefasciatus*; however, anopheline mosquitoes are important vectors in rural areas. The other major filarial parasite of humans transmitted by mosquitoes is *Brugia malayi*, which is patchily distributed in India and Southeast Asia. Mosquito-borne filariasis, widely known for the disfiguring elephantiasis which occurs in a proportion of cases, is still a major public health problem in the tropics. Considerable research effort has been expended on developing improved methods of chemotherapy, on immunodiagnosis and on understanding the factors that influence transmission by mosquitoes.

9.1.1 Choice of parasites and vertebrate hosts for experimental studies

Experimental studies with *W. bancrofti* are made difficult by the absence of a convenient laboratory host for the vertebrate stages of the parasite. Consequently, studies with *W. bancrofti* typically require a laboratory in or near an endemic area of the tropics or subtropics and the cooperation of a human volunteer, either for donated blood or for direct feeding of mosquitoes. In the case of the nocturnally periodic form of the parasite (the most widespread), blood collection and direct feeding must normally be carried out at night. The donated blood can then either be used directly in a membrane feeder unit or cryopreserved for subsequent use. Because of the difficulties of work with *W. bancrofti*, *B. malayi* and the related parasite *B. pahangi* have been widely used in experimental studies. For these species, adult worms can readily be maintained in Mongolian jirds, *Meriones unguiculatus* (Ash and Riley, 1970).

B. malayi exists in at least two forms: one nocturnally periodic which is not usually zoonotic; the other nocturnally subperiodic, which has primates and carnivores as natural reservoirs. These parasites have different natural mosquito vectors. The most widely used isolate of *B. malayi* is a subperiodic parasite.

The original experimental hosts for *B. malayi* and *B. pahangi* were domestic cats; dogs and ferrets (domesticated form of *Mustella putorius*) can also be readily infect-

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ed. For model studies of aspects of the vertebrate host response, cats or dogs may still be preferred hosts; both can be found naturally infected in the wild, and both readily develop a prolonged and high microfilaraemia. Furthermore, the development and location of the parasites within the host are similar to that in humans, unlike in rodents. However, for both ethical and practical reasons, in studies concerned with the insect vector, their use has largely been abandoned.

Although many rodents are relatively refractory to infection, the Mongolian jird is highly susceptible and is the most commonly used laboratory maintenance host. Jirds are relatively easy to handle and maintain in the laboratory and have a lifespan of 2 years or more, factors that contribute to their suitability as hosts. Infections in jirds can be produced by subcutaneous inoculation, producing a microfilaraemia (Ash and Riley, 1970), or by interperitoneal injection (McCall *et al.*, 1973). The latter is particularly useful, since microfilariae can easily be harvested by peritoneal lavage.

Blood-derived microfilariae are normally obtained from the retro-orbital sinus, a much more troublesome procedure. Furthermore, when microfilariae are obtained from the peritoneum they can be diluted by resuspension in blood, allowing mosquitoes to be fed on a controlled number of parasites. Microfilariae from the blood tend to have a higher subsequent infectivity to mosquitoes than those from the peritoneal cavity (Schrater *et al.*, 1982), but for routine purposes the ease with which large numbers of microfilariae can be harvested outweighs this disadvantage.

Following inoculation, the mean prepatent period for subcutaneously inoculated *B. pahangi* is 67 days, and most animals become patent by 84 days (Ash and Riley, 1970). The patency period is similar in *B. malayi* and is about 20 days longer for interperitoneally infected jirds; by 100 days most infections have become patent. Typically, 70–100 infective larvae are used to infect each jird; lower numbers may increase the proportion of nonpatent infections. Ash and Riley (1970) reported higher worm recoveries of *B. pahangi* from male jirds. For laboratories that are just establishing *Brugia* infections, it is no longer necessary to ship infective-stage larvae (L3s) in liquid nitrogen or transfer infected jirds from other laboratories. Yates *et al.* (1994) have demonstrated that L3s may be kept in nutrient medium at 20 or 37°C and remain infective for up to 120 h, with no detectable drop in infectivity after overnight storage. It has thus been possible to ship L3s overnight by air and retain 99% viability. Infective larvae stored overnight at 0°C are unable subsequently to survive in jirds.

Dirofilaria species are commonly used in laboratory studies of the mosquito–filaria interaction, particularly the parasites of domestic dogs, *D. immitis* (canine heartworm) and *D. repens*. The techniques described for mosquito infection by membrane feeding and harvesting L3s can be applied to them; however, it should be remembered that in the mosquito the site of development of *Dirofilaria* is the Malpighian tubules, whereas *Brugia* and *Wuchereria* develop in the flight musculature.

9.1.2 Choice of mosquitoes for experimental studies

While the natural vectors of *Brugia* are mainly *Anopheles* or *Mansonia* species, *Aedes* mosquitoes are more convenient laboratory hosts. A great deal is known of the biology of *Ae. aegypti*, the most widely used laboratory host of *Brugia*. Furthermore, reasonably detailed genetic maps are available, based on conventional genetic crosses

(Munstermann, 1990). It should be noted, however, that *Ae. aegypti* is not known to be a natural vector of *Brugia* or *W. bancrofti*. Most field populations are highly refractory to infection with *Brugia* (Rodriguez and Craig, 1973); however, some sylvan East-African strains are susceptible to both *Brugia* (Paige and Craig, 1975) and *W. bancrofti* (Kilama, 1976).

For laboratory transmission, a stock of *Ae. aegypti* homozygous for the f^m allele is necessary (Macdonald and Ramachandran, 1965) (Note 1). In addition to *Ae. aegypti*, other *Stegomyia* species (ie. *Ae. polynesiensis* and *Ae. pseudoscutellaris, Ae. cooki*, and *Ae. tabu)* are susceptible to both *Brugia* and *Wuchereria* (Macdonald, 1971; 1976), while *Ae. mediovittatus* may also serve as a suitable host for susceptibility studies (Trpis, 1994).

Macdonald (1971) has reviewed methods for maintaining mosquito vectors of filariasis. Additional references may also be found in Chapters 1 and 2.

9.1.3 Techniques

The techniques described here concentrate on *Brugia* and include: (i) infection of jirds and subsequent harvesting of microfilariae; (ii) infection of mosquitoes feeding and harvesting of infective larvae; (iii) infection of mosquitoes by intra-thoracic inoculation; (iv) transfer of infective larvae between laboratories; and (v) cryopreservation of microfilariae.

9.2 MATERIALS

9.2.1 Infection of jirds and subsequent harvesting of microfilariae

1. Mongolian jirds (*Meriones unguiculatus*), 6 weeks old.
2. Infective-stage larvae (L3s) of *Brugia* in infected mosquitoes.
3. Hayes saline: 0.15 M NaCl, 1.8 mM $CaCl_2$, 2.7 mM KCl, 1.2 mM $NaHCO_3$.
4. TC199 medium (commercially available).
5. Hank's balanced salt solution (HBSS): 5.4 mM KCl, 0.44 mM KH_2PO_4, 0.8 mM $MgSO_4$, 0.14 M NaCl, 4.2 mM $NaHCO_3$, 0.33 mM Na_2HPO_4, 5.6 mM glucose, 17 mg/L phenol red. (Can be prepared or obtained commercially). Refrigerate at 4°C.

9.2.2 Infection of mosquitoes by membrane feeding and harvesting of infective larvae

1. Hayes' saline: (Section 9.2.1).
2. Microfilariae (mff) of *Brugia*.
3. Badrouche membrane, or chicken skin membrane from washed and plucked skin of 10–14 day-old chickens.
4. Water-jacketed glass feeding unit, with or without stirrer (Figure 9.1).
5. Blood containing 10 IU heparin/mL (rabbit blood is suitable) for suspension of mff.
6. Hank's balanced salt solution (HBSS): (Section 9.2.1).

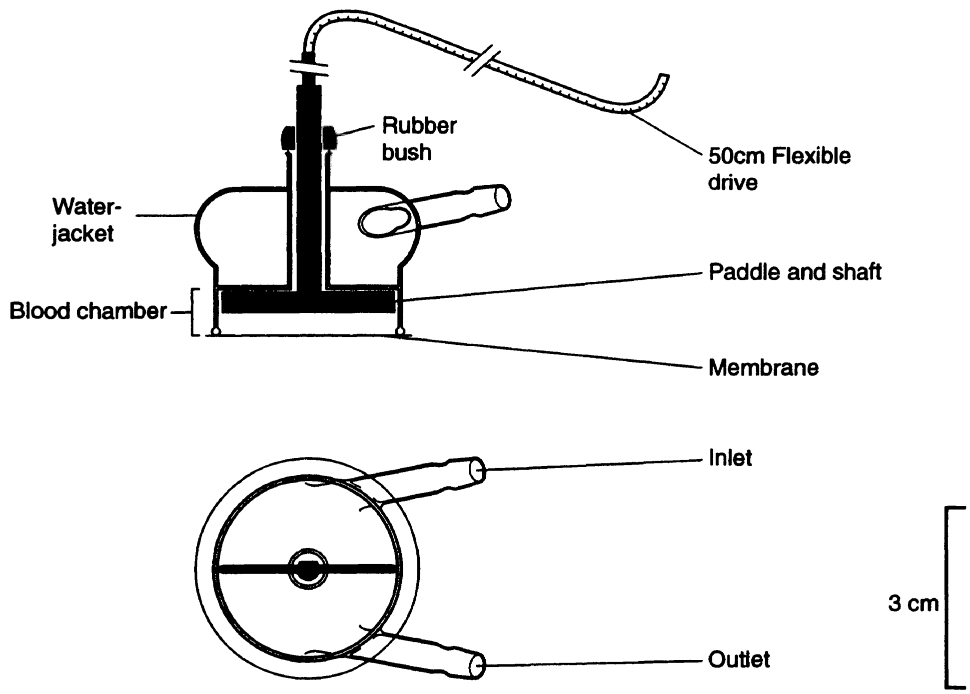


Figure 9.1 A membrane feeding unit fitted with a stirring device to prevent sedimentation of red blood cells and microfilariae. (From Wade, 1976.)

7. Ham's nutrient mixture F-12, with added amphotericin-B (0.5 μg/mL), 100 U/mL penicillin and 100 μg/mL streptomycin. (Commercially available). Refrigerate at 4°C.
8. Heat-inactivated fetal calf serum (FCS).

9.2.3 Infection of mosquitoes by intrathoracic inoculation

1. Glass capillary needle drawn to 60–80 μm tip diameter.
2. Suction air-line connected to capillary tubing to hold mosquitoes.
3. Hayes' saline: (Section 9.2.1), containing sodium penicillin G (50 U/mL), streptomycin sulfate (50 μg/mL) and nystatin (100 μg/mL).
4. Freshly harvested microfilariae.

9.2.4 Cryopreservation of microfilariae

1. Low molecular weight hydroxyethyl starch (HES)(c.150 kDa): 40% (w/v) solution in 0.15 M NaCl (Note 2).
2. Liquid nitrogen refrigerator fitted with a polystyrene holding plug to hold up to ten 2 mL capacity plastic cryopreservation standard ampoules. By varying the height of the plug in the neck of a 35 L liquid nitrogen refrigerator, the rate of cooling of samples can be controlled.
3. Thermocouple to monitor rate of cooling.

9.3 METHODS

9.3.1 Infection of jirds with *B. malayi* or *B. pahangi*

1. If mosquitoes are maintained at around 26–27°C, *B. pahangi* reaches the infective stage at 9–10 days following infection. Leave mosquitoes until 11–12 days to allow maximum recovery of L3s.
2. Methods for harvesting L3s are described in Section 9.3.2.
3. Accumulate groups of approximately 100 L3 larvae for each jird to be infected in TC199 medium (Note 3).
4. Draw the L3s into a 2 mL syringe fitted with a 23 G needle.
5. Hold the jird firmly by the loose skin on its back, and with its head held slightly down, insert the needle into the peritoneum through the lower lateral aspect of the ventral surface of the abdomen. Expel the L3 parasites from the syringe. The jird need not be anaesthetized prior to inoculation.
6. After 90–100 days, examine jirds for presence of microfilariae in peritoneal cavity. To do this, draw 1–2 mL of HBSS into a syringe fitted with a 23 G needle. With the jird lightly anaesthetized with a halothane/oxygen gas mixture (Note 4), hold it slightly head-down and insert the needle into the peritoneal cavity as before and expel syringe contents. Without withdrawing the needle, slightly elevate the jird and after approximately 15 s, refill syringe. Should there be any resistance due to tissue blocking the needle, slightly depress the syringe plunger before attempting to refill.
7. Place 20 μL of the withdrawn fluid on a microscope slide and cover with a plastic cover glass. Observe under a compound microscope with 10× objective and 10× eyepiece, and where necessary count microfilariae. Alternatively, use the counting chamber technique described in Section 9.3.2a.

9.3.2 Infection of mosquitoes

(a) Direct feeding on the host

Membrane feeding is probably the most convenient method for producing controlled filaria infections in mosquitoes and is greatly to be preferred to direct feeds. However, for some purposes it may be necessary to feed mosquitoes directly on an infected animal host, for example, where it is necessary to study the process of infection of the mosquito, or the process of passing on the infection, in an environment as close to the natural as possible (Lindsay and Denham, 1986).

Feeding may be on an infected cat, dog, or jird following anaesthetization of the animal with a suitable agent. Clearly, the infection must have established a patent microfilaraemia of sufficient intensity and this is not as predictable a system as obtaining mff from the peritoneum of the jird. Blood samples are most commonly taken from an ear vein or the femoral vein of the cat, or from the periorbital sinus of the jird.

The following description relates to the use of the cat. Handling procedures and anaesthetics will vary according to the host and should be checked with the local veterinarian. Anaesthesia accompanied by direct feeding on cats requires experienced technical support and should not be attempted by untrained individuals. Indeed in many countries laws governing animal experimentation will severely restrict the procedures to be followed. For many people the domestic cat is a much-loved pet and such procedures should not be carried out unless absolutely necessary for the advancement of science in the cause of the alleviation of human suffering.

(i) Counting microfilariae

Before planning a feed, check parasitaemia in the animal by taking a blood sample, usually from a nick in the large ear vein. With care, this sample can be taken without the need to anaesthetize the animal. If a precise count is not required, 20 μL samples of blood can be covered with a coverslip and examined at 100× in a compound microscope and the moving parasites roughly counted. For a more accurate assessment of the mff load use one of the following two methods:

1. Take 40–60 μL of blood and make a thick blood smear by spreading the blood evenly and quickly over an area roughly 1.5 × 4 cm on a microscope slide. Allow to dry at RT in a dust-free environment.
2. Once dry, immerse the slide, on edge, for 30 min in a 1:6–1:10 dilution of Giemsa concentrate in phosphate buffered water pH 7.2. The smear will both dehaemoglobinize and stain at the same time. Allow the smear to dry then cover with immersion oil and a glass coverslip and examine under a compound microscope with a 10× eyepiece and a 10× objective. A mechanical stage is necessary for accurate counting.

Alternatively (based on Denham *et al.*, 1971; Southgate, 1973) (Note 5):

1. Take a counting chamber with a well 15 mm × 45 mm × 1 mm. Pieces of microscope slide mounted on another slide with a strong waterproof adhesive (e.g. Araldite) are ideal for this purpose. Ideally the slide that forms the floor of the chamber should be scored with lines roughly 2 mm apart before glueing together. The lines aid counting.
2. Add 0.5 mL tapwater and then 60 μL of blood. Mix thoroughly to induce haemolysis.
3. Examine chamber contents and count mff under a dissecting microscope at 50×.

(ii) Blood-feeding

1. Cats should be fasted for 12 h prior to anaesthesia to minimize the risk of vomiting during induction or recovery. Consult your local veterinarian on the most appropriate locally available anaesthetic. Intramuscular ketamine (20 mg/kg) normally produces an adequate level of anaesthesia but its low pH makes the injection somewhat painful.
2. Once the animal is under anaesthesia, shave the side of the abdomen using clippers and then lay it on the cage of mosquitoes to be fed (Note 6). Protect the cat's head so that mosquitoes cannot feed on the face. The animal should be monitored throughout the feed as duration of anaesthesia may be variable.
3. Once feeding is completed the animal should be monitored until it is well recovered from the anaesthesia.
4. Because of the anaesthetic and the need for fasting, animals must be rested for at least 2 weeks between blood-feeding.

(b) Membrane feeding

Membrane feeding allows mff from the jird peritoneum to be fed to mosquitoes at a controlled concentration. Apart from the convenience of the jird host, the variance in the mean infection is reduced compared with direct donor feeding, making this more convenient for experimental studies that look for changes in infection rate (Wade,

1976). Mosquitoes are most readily fed 4 days following emergence, after being deprived of access to water or sugar solution overnight.

1. Draw 2 mL of HBSS into a syringe fitted with a 23 G needle. With the jird lightly anaesthetized with a halothane/oxygen gas mixture (Note 4), hold it slightly head-down and insert the needle into the peritoneal cavity. Without withdrawing the needle, slightly elevate the jird and after approximately 15 s, refill syringe. Should there be any resistance due to tissue blocking the needle, slightly depress the syringe plunger before attempting to refill.
2. Transfer syringe contents to a sterile universal bottle.
3. Examine 2 × 20 µL samples of the withdrawn fluid on a microscope slide, covered with a coverslip. Count mff under a compound microscope with 10× objective and 10× eyepiece.
4. Adjust mff concentration to required level by mixing peritoneal lavage with blood in appropriate ratio. For purposes of routinely infecting mosquitoes, 10 mff/µL of blood will provide a mean infection of approximately 10 infective larvae per mosquito. Higher concentrations may be used, but above 10 mff/µL, excessive mortality of mosquitoes may occur. Heparinized blood (10 IU heparin/mL) from a rabbit is suitable for making the necessary concentration adjustments.
5. Transfer blood into feeder unit fitted with membrane, using a syringe fitted with a long needle which has been bent slightly to facilitate access through top. Circulate water at 37°C and place unit on top of netting of container holding mosquitoes.
6. Feeding should be completed within 30 min. Up to 200 mosquitoes can be infected at a time using this technique. Transfer of fed mosquitoes is best carried out several hours after feeding to avoid undue damage but with care can be done immediately after the feed.
7. Maintain fed mosquitoes for approximately 10 days at 26°C and 80% RH. Provide access to 10% sucrose solution from the second day and provide a bowl for egg-laying, so that mosquitoes are not full of eggs when the time comes for dissection (Note 7).

(c) Recovery of infective larvae

Optimal recovery of infective larvae usually occurs when mosquitoes are dissected on 11–12 days after infection.

(i) For small numbers of mosquitoes

1. Dissect individual mosquitoes in Hayes saline and, where infective larvae are required, using a fine-tipped glass Pasteur pipette transfer L3s to an embryological block or Petri dish containing appropriate saline (Note 3).

(ii) For large numbers of mosquitoes

Use the modified Baermann technique (Note 8):

1. Infected mosquitoes are immobilized by chilling, placed on a glass plate and crushed by rolling a glass tube over them.
2. The debris is then immediately washed with HBSS warmed to 27°C into a Baermann funnel lined with several layers of cotton gauze. A wire screen can be used to support the gauze (Note 9).

3. Immediately add sufficient warmed HBSS to the funnel to cover the macerated mosquitoes and leave at room temperature for 60 min. L3s settle at the bottom of the funnel and can be drawn off.

(d) Transfer of infective larvae between laboratories

L3s can be kept overnight at 20 and 37°C in Ham's nutrient mixture F-12, with antibiotics and 1% heat-inactivated fetal calf serum (FCS), with virtually no drop in infectivity and may be shipped to other laboratories in this medium (Yates *et al.*, 1994). If this is to be done, it is possible to use Ham's F-12 medium plus antibiotics for separation of the L3s from the mosquitoes, 1% FCS being added subsequently to the medium containing the L3s.

9.3.3 Infection of mosquitoes by intrathoracic inoculation

This is a useful technique where the numbers of microfilariae entering the mosquito needs to be strictly controlled for quantitative comparisons (Jin and Xu, 1991) and has been used in studies of the haemolymph response to infection (Townson and Chaithong, 1991). It also provides a means of avoiding the influence of gut passage and can be used to infect male mosquitoes, although the factors limiting filarial development may be quite different in males than in females (Terwedow and Rodriquez, 1973; Townson, 1974). The procedures described here are based on Townson (1974) and Townson and Chaithong (1991). Using them, survival rates greater than 85% over 10 days can be achieved in *Ae. aegypti*. Survival rates are somewhat lower in *Anopheles* and *C. quinquefasciatus*.

1. Glass capillary tubing is drawn out in a flame to a tip diameter of 60–80 μM. Movement of the capillary needle is controlled by a micromanipulator.
2. Mosquitoes for inoculation should be between 2–4 days after emergence. They may be held by suction on the tip of a glass capillary connected to a vacuum line, or simply placed in a dish following CO_2 anaesthesia (Chapter 11).
3. Microfilariae are suspended in Hayes saline to which has been added sodium penicillin G (50 U/mL), streptomycin sulfate (50 μg/mL) and nystatin (100 μg/mL). In the absence of antibiotics, survival can be poor but tetracycline should be avoided as it can interfere with the subsequent development of filariae.
4. Microfilariae are taken up in the glass capillary needle while being viewed at 40× under a dissection microscope and the numbers counted.
5. The needle is then inserted into the membraneous area below the paratergite on the lateral aspect of the thorax and the saline and mff expelled. Up to 10 mff can be inoculated per mosquito in a volume not exceeding 2 μL (Note 10).
6. By replacing the glass needle with an electrolytically sharpened tungsten needle, L3s can be experimentally introduced into the abdomen of mosquitoes

9.3.4 Cryopreservation of microfilariae

Cryopreservation provides a technique whereby laboratories which are remote from endemic areas of filariasis can work with parasites of humans. The viability of mff preserved in liquid nitrogen will not compare with that of fresh mff and hence this technique should be used only when there is no suitable laboratory animal host avail-

able. Many methods can be found in the literature and numerous cryoprotectant chemicals have been utilized, with varying success.

The following method is based on Minjas and Townson (1980) and uses hydroxyethyl starch (HES) as cryoprotectant. HES has been used extensively in trials for cryopreservation of human erythrocytes and has the advantage that it is nontoxic to mff and to mosquitoes fed on cryopreserved parasites. By contrast, dimethyl sulfoxide (DMSO), another commonly used cryprotectant, is toxic to mff at temperatures above freezing and must be rapidly separated from mff after thawing. Furthermore, traces of DMSO in blood being fed to mosquitoes are highly toxic. Cryopreserved microfilariae have proved useful in assessing whether aposymbiotic *C. quinquefasciatus* can support development of *W. bancrofti* (Curtis *et al.*, 1983).

1. Blood samples with microfilariae (mff) are collected by venepuncture and heparin added to 10 IU/mL.
2. Hydroxyethyl starch (HES) dissolved in 0.15 M NaCl to a concentration of 40% w/v, is mixed with blood containing mff to a final concentration of 16 g/100 mL.
3. The mixture is held in ice/water for 15–30 min before being dispensed into 2 mL capacity plastic ampoules for cryopreservation.
4. Batches of 10 ampoules at a time are placed in a holding plug constructed from expanded polystyrene which in turn fits into the neck of a 35 L liquid nitrogen refrigerator. The rate of cooling is controlled by adjusting the height of the plug in the neck, temperatures being monitored by means of a thermocouple inserted into one ampoule for each batch being frozen.
5. Mff are cooled at a rate of c. 1.5°C/min down to –55°C, followed by plunge into liquid nitrogen. Ampoules may then be transferred rapidly to the liquid nitrogen refrigerator.
6. When mff are required for infection of mosquitoes, ampoules are removed and transferred rapidly to a shaking water bath at 37°C, thawing being complete within 1 min. Mff may be held at 37°C for 30 min to allow full motility to be restored, before feeding to mosquitoes.
7. If excessive haemolysis has occurred, before mff are fed to mosquitoes, additional blood can be added, provided that the density of mff remains within the target for the experiment.

NOTES

1. The strain designated SS by Macdonald and Ramachandran (1965) is a highly susceptible strain, homozygous for f^m and with wild-type eye colour. The strain supplied to other laboratories by the Liverpool School of Tropical Medicine (LSTM) for filaria transmission has most commonly been a recombinant homozygous susceptible (f^m/f^m) stock marked with the recessive eye colour mutant *re* (red-eye) (Macdonald and Sheppard, 1965). In the literature this is the strain most commonly referred to as the 'Liverpool' strain, although in LSTM, the designation 'Liverpool' has always applied to an unselected laboratory stock from which the f^m mutant was originally selected.
2. High molecular weight HES is equally protective against freezing damage but more viscous in solution and hence more difficult to handle.

3. A variety of media have been used to suspend L3 larvae for inoculation into jirds. The constitution of this medium is relatively unimportant for short-term survival of L3s, since jirds may be infected orally with water into which L3s have escaped (Bosworth *et al.*, 1976).
4. Halothane is not recommended for use by pregnant female laboratory workers.
5. Up to 30% of microfilariae may be lost from blood smears during staining, more if glass slides are not scrupulously cleaned. The counting chamber method tends to give more accurate results.
6. Normally *Ae. aegypti* females are best infected at around 4 days post-emergence.
7. Oviposition is associated with a slightly increased mortality.
8. This approach is especially useful when large numbers of animals are to be infected.
9. Crans (1971) has described a chamber for separating L3s.
10. Klowden (1981) has described a technique whereby mff can be introduced directly into the mosquito gut by enema.
11. Regarding health and safety aspects of work with filariae, both *B. malayi* and *B. pahangi* are capable of infecting humans, although the latter would not be expected to produce a patent infection. While these conditions are not life threatening, they can produce fever and enlarged and tender lymph nodes, together with a persistent lymphoedema. The earliest symptoms, usually lymphangitis, are not normally experienced until some 6 months after infection. Hence, when handling infections in mosquitoes, in addition to the normal precautions taken to prevent mosquito escapes (Gerberg *et al.*, 1994), special measures must be taken to prevent accidental transmission. In many countries there will be laws and regulations governing the conditions under which such experiments should be conducted.

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CHAPTER TEN

Infection of phlebotomine sandflies with Leishmania

Paul A. Bates

10.1 INTRODUCTION

Species of sandflies that are capable of transmitting leishmaniasis are found in two genera: *Lutzomyia* and *Phlebotomus*. Only the females blood-feed and transmit disease *via* their bite, *Lutzomyia* species in the Americas, and *Phlebotomus* spp. elsewhere. Under natural conditions each species of sandfly is usually the vector for a single species of *Leishmania*. In female sandflies *Leishmania* parasites exist as extracellular flagellated promastigote forms and are found exclusively in the alimentary canal. In the majority of cases the parasites cycle between their sandfly hosts and certain wild mammals, with human infection a zoonosis. However, there are some important exceptions in which there is a human host–sandfly cycle; visceral leishmaniasis in India, for example. In their mammalian hosts *Leishmania* parasites exist as nonmotile intracellular amastigote forms in tissue macrophages. The parasites cause two main types of human disease: visceral leishmaniasis and cutaneous leishmaniasis. Since the taxonomy of both phlebotomine sandflies and *Leishmania*, and their host–parasite relationships, are all subjects of current research, the precise number of naturally occurring parasite–sandfly combinations remains to be determined. Currently there are approximately 70 proven or suspected vectors of leishmaniasis (Lane, 1993) and 20 or so recognized species of *Leishmania* (Lainson and Shaw, 1987).

10.1.1 Vector–parasite combination

Faced with a variety of possible combinations, one of the most important decisions to be made when embarking on experimental infection of sandflies is the choice of sandfly and parasite species to be used, both of which are strongly influenced by the facilities available to the investigator. For the sandfly, an insectary with an established healthy laboratory colony is required for most types of experimental work. For the parasite, *Leishmania*-infected animals and/or culture facilities for maintaining parasites are necessary. A selection of combinations that have been used is shown in Table 10.1. These include both natural combinations, i.e. those in which the sandfly species is a proven vector of the specific parasite, and those in which an artificial

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combination is used. A detailed discussion of the validity of all possible combinations is not possible here, and also critically depends on the specific purpose of the experiment, but some guidelines can be given. In general most investigators wish to reproduce normal infections, in which case natural combinations are preferable. Where this is not possible, sandflies that are capable of sustaining full cyclical development of the parasite should be used, i.e. production of mammal-infective metacyclic promastigotes. An example of a permissive species of fly that can support development of a number of other *Leishmania* species, in addition to that which it naturally transmits, is provided by *Lutzomyia longipalpis*, the vector of *Leishmania chagasi* (Table 10.1). If in doubt, a good test of whether cyclical transmission can occur is to determine whether an infected fly can transmit disease to a suitable experimental animal host *via* bite, usually a mouse or hamster (Killick-Kendrick, 1986). One important exception to these guidelines is where the object of the exercise is to understand barriers to parasite development, in which case infections in unsuitable sandfly hosts may be quite informative. Despite the large choice of vector–parasite combinations the methods used in each case are similar.

10.1.2 Infection methods

A further strategic choice is between the two basic methods that can be used for the infection of phlebotomine sandflies with *Leishmania*: feeding on infected animals and artificial membrane feeding. This can also affect the choice of vector–parasite combination, since feeding on infected animals is possible practically only for those species of *Leishmania* causing cutaneous disease. Theoretically it is also possible to infect flies by feeding on animals with visceral disease. However, the infection rates are too low for most experimental purposes. Artificial membrane feeding can be used for all

Table 10.1 A selection of vector–parasite combinations that have been used for experimental infection of phlebotomine sandflies. These include vectors for the most frequently studied species of *Leishmania*. The actual number of combinations that have been used or are possible is much larger than is shown here

Sandfly species	*Parasite species*	*Type of disease*	*Natural or artificial combination*
Lu. longipalpis	*Le. chagasi*	Visceral	Natural
Lu. longipalpis	*Le. mexicana*	Cutaneous	Artificial
Lu. longipalpis	*Le. amazonensis*	Cutaneous	Artificial
Lu. longipalpis	*Le. braziliensis*	Cutaneous	Artificial
Lu. trapidoi	*Le. panamensis*	Cutaneous	Natural
Lu. wellcomei	*Le. braziliensis*	Cutaneous	Natural
P. argentipes	*Le. donovani*	Visceral	Natural
P. ariasi	*Le. infantum*	Visceral	Natural
P. dubosqui	*Le. major*	Cutaneous	Natural
P. papatasi	*Le. major*	Cutaneous	Natural
P. perniciosus	*Le. infantum*	Visceral	Natural
P. sergenti	*Le. tropica*	Cutaneous	Natural

species of parasite and has another potential advantage, that the concentration of infecting parasites and the other contents of the blood meal can be independently manipulated.

Two other variables that need to be considered in the choice of infection method are the feeding rate and the infection rate. The feeding rate is the percentage of female flies in the cage that take a blood meal. The infection rate is the percentage of blood-fed flies that subsequently develop infections. Both of these can be affected by the choice of infection method. Unfortunately, when embarking on such work for the first time or switching to a new parasite–vector combination it is not possible to make precise predictions for either of these variables. These need to be empirically determined before any major experiments are attempted. The feeding rate is generally higher using an infected animal than with membrane feeding – the presence of a live animal is a strong stimulus for blood-feeding. However, the infection rate is more unpredictable when using an animal. Factors that can influence success are the shape and size of the lesion and the effectiveness of masking other parts of the body. If these are optimized high infection rates can also be achieved. Nevertheless, there remains an underlying variability with feeding on animals, as each fly may feed at a different site on the lesion where there are local variations in the density of parasites below the skin. Although consistently high rates of feeding are more difficult to achieve with artificial membranes, the infection rate is more predictable. The flies ingest a standardized concentration of parasites, which is normally a sufficiently high concentration to ensure that virtually all blood-fed flies become infected.

Whichever method is chosen, it is important to remember that virtually all species of *Leishmania* that are used experimentally are capable of causing human infection and, therefore, experimental procedures should be carried out in accordance with statutory safety regulations and good practice as detailed below. Much useful information on the biology of *Leishmania* has been gained from the study of such experimental infections (for examples, Schlein, 1993; Walters, 1993). These methods will be of continuing importance in functional studies at the molecular level for both partners in the vector–parasite relationship.

10.2 MATERIALS

10.2.1 Feeding on infected animals

1. Source of sandflies. The most convenient source is a laboratory colony (Chapter 3) but wild-caught flies could also be used if available. For experimental reproducibility a laboratory colony is preferred and is essential for any long-term studies. However, certain species of sandfly have not been successfully colonized and if, for example, the purpose of the experiment is a test of transmission in a vector incrimination study, wild-caught flies may be preferred. However, additional expertise in the identification of sandflies is required. Methods for the capture of sandflies will not be described here but the interested reader should consult Lane (1993).
2. *Leishmania*-infected animals. These are normally laboratory animals infected with species of *Leishmania* that cause cutaneous disease, but wild reservoir hosts or domestic dogs with generalized leishmaniasis could also be used. Laboratory hosts for cutaneous leishmaniasis are usually mice (e.g. BALB/c) or golden ham-

sters (*Mesocricetus auratus*). Experimental infection is by intradermal inoculation of parasites into a suitable site such as the shaven rump or footpad. Lesions take from one to several months to develop, depending on the parasite species and inoculum. For further details consult Bray (1987).

3. Suitable insectary facilities. A separate room where infections are performed and infected flies maintained is preferred. This prevents the accidental mixture of infected flies with the main breeding colony. A dedicated room also prevents accidental exposure to those not directly concerned with the study and makes recovery of any escaped flies easier.
4. Supplies of double-netted cages, small glass vials, lids, netting, filter paper, plastic boxes, cotton wool and sugar solution for feeding. Apart from the double-netted cages, these are standard items required for colony maintenance (Chapter 3 for further details). Double-netted cages are made to the same design as those used in routine colony maintenance, but are simply made with two layers of netting. They should be used to keep groups of *Leishmania*-infected sandflies. The extra barrier provides insurance against escape through small undetected holes in the netting. An escaped uninfected fly is an irritation; an escaped *Leishmania*-infected fly is a serious safety risk.
5. Covering material. There is some laboratory evidence that flies show a feeding preference for lesions when given free access to an infected animal (Coleman and Edman, 1988), although infection rates were not determined in this study. However, to maximize the infection rate it is useful to have some form of material that can be used to screen off those parts of the animal's body that provide alternative feeding sites to the lesion. This can be simply provided by polythene sheeting, thick cloth, micropore surgical tape, or by placing the animal in a suitable-sized plastic tube or chamber with a hole in the wall to provide access to the lesion.
6. Sandfly dissection materials. Fine mounted needles, microscope slides, dissecting microscope, sterile Locke's solution: NaCl 9.2 g/L, $CaCl_2$ 0.24 g/L, $NaHCO_3$ 0.15 g/L, KCl 0.42 g/L, glucose 1.0 g/L, ddH_2O.

10.2.2 Artificial membrane feeding

1. Source of sandflies – see above.
2. Source of parasites. There are two sources of parasites for membrane feeding: those isolated from infected animals and those obtained by *in vitro* culture. Experimental infection of animals with parasites causing cutaneous disease has been dealt with above (Section 10.2.1, step 2). For those causing visceral disease, infection is usually by intraperitoneal or intravenous injection (Bray, 1987). Mice, hamsters or cotton rats (*Sigmodon hispidus*) are commonly used hosts and the parasites are subsequently isolated from the spleen or liver. For membrane feeding, amastigotes isolated from infected animals are the most frequently used source of parasite and are the stage in the life cycle that initiates sandfly infection under natural conditions. If desired they can be cryopreserved in 8% dimethyl sulfoxide/culture medium, thawed and washed prior to usage. For references to the variety of amastigote isolation methods see Bates *et al.* (1992). *In vitro* culture provides some alternative sources of parasites that may be more convenient and minimize the use of infected animals. Macrophages or

macrophage-like cell lines can be used as host cells for *Leishmania in vitro* and have been used directly in membrane feeding (Tesh and Modi, 1984). Parasites can also be cultured axenically, i.e. without a host cell, as promastigotes or amastigotes. Axenic culture of promastigotes is a routine procedure in most *Leishmania* laboratories. For a review of promastigote culture methods and culture media see Evans (1987). If using promastigotes, it is best to use material of low subpassage (<10) and in the logarithmic phase of growth. Axenic amastigote culture is a more specialized technique and currently is only available for a limited number of strains and species (Bates, 1993). Axenic amastigotes provide a useful compromise, providing the appropriate stage of the life cycle but avoiding the need for macrophage cultures. Parasites are counted using an Improved Neubauer haemocytometer. Amastigote samples can be counted directly; promastigote samples should be immobilized first by mixing with an equal volume of 2% formaldehyde in phosphate-buffered saline.

3. Feeding apparatus. Various devices have been used for membrane feeding sandflies but all have the same general design (Ward *et al.*, 1978; Note 1). These are similar to the glass feeders used for other haematophagous arthropods. Since such devices are not commercially available, access to a competent glassblower is invaluable. A waterbath capable of delivering 30–37°C together with tubing to connect to the inlet and outlet tubes of the feeder are required. Cotton thread and micropore tape are required to attach the membrane to the feeder.
4. Membranes. Many different membranes have been tried but the most commonly used are skins derived from 1–5 day-old chicks (Ward *et al.*, 1978; Note 2). The chicks are stored frozen at –20°C until required. To prepare a membrane an animal is first thawed out. The best area to use is the skin overlying the breast region. The feathers need to be removed which can be done before dissection, after dissection or later when the membrane is attached to the feeder. The skin is carefully dissected away from the body taking care not to tear it. It is washed first with 70% ethanol and then with sterile distilled water. If desired, the skin can be wrapped in silver foil and placed in a plastic bag for further storage at –20°C. Otherwise it is ready for use as described below (Section 10.3.2, step 2).
5. Fresh blood. Sterile rabbit blood is commonly used. To prevent problems with clotting this is either heparinized or defibrinated by shaking with small glass beads for 5 min. Depending on the source of parasites to be used the blood may also be heat-inactivated by incubation at 56°C for 30 min to destroy complement activity. This is necessary for cultured promastigotes, which are serum sensitive, but not amastigotes. Some lysis of the erythrocytes may result. An alternative option is to spin out the erythrocytes and heat-inactivate the plasma only, then reconstitute the mixture. These are personal preferences and do not appear to have a significant effect on feeding success.

10.3 METHODS

10.3.1 Feeding on infected animals

1. Prepare a double-netted cage containing a suitable number of female flies. The number depends on the purpose of the experiment, any subsequent statistical analysis of the results that is planned, and the predicted feeding/infection rates.

A typical experiment may use 100–200 female flies with 2–4 *Leishmania*-infected mice. It is common practice to include a small number of male flies, as their presence may encourage the females to feed. The cage can be prepared a day or two in advance. The flies may be anything from one to several days old. However, younger flies are preferred as older flies will not survive as long after blood-feeding or oviposition (if at all). A useful safety precaution is to count in the number of flies so that all can be accounted for later (step 5). The sugar meal is removed the day before infection to ensure that the flies are hungry.

2. Anaesthetize the *Leishmania*-infected animal(s), usually a mouse or hamster with a prominent nodular lesion of 1 cm or greater in diameter. Sufficient anaesthetic should be used to ensure that the animal remains asleep for greater than 1 h. This procedure should be carried out in accordance with national regulations governing the use of animals in experiments.
3. The animal is placed in the cage with the sandflies. To maximize the infection rate, feeding should be confined to the lesion, and other parts of the animal where the flies may like to feed, such as the ears, limbs and tail, should be made inaccessible. Sandflies are unable to feed through the fur of a mouse or hamster. With rodent hosts screening can be achieved by using polythene sheeting or a thick cloth with a hole for access to the lesion. Alternatively, a chamber or tube can be used. Small areas can be screened using micropore surgical tape. Whichever method is used the comfort of the animal must be periodically checked. If the animal is confined it is essential that it is able to breathe freely whilst under anaesthesia (Note 3 gives an alternative method).
4. After a suitable period of time the animal is removed from the cage. Normally 1 h is sufficient for most of the females to feed. During periodic checking of the animal (above) the number of flies still feeding can be noted. After feeding the females rest on the sides and roof of the cage. During removal of the animal it is important to take care to brush off all the flies. This is achieved by carefully pulling the animal through the netting sleeve in the palm of the hand, closely drawing the netting over the body as it is removed. It is useful to secure the netting sleeve around the arm with one or two elastic bands to discourage flies from escaping. For those inexperienced in the technique, placing the animal temporarily in another empty cage is another wise precaution. This allows any further flies trapped in the fur to come off.
5. The flies are then individually tubed up in small glass vials and counted (Note 4). All female flies should be treated as potentially *Leishmania*-infected whether it is obvious from their red distended abdomen that they have taken a blood meal or not. Normally only blood-fed flies are included in any subsequent experiment or analysis. However, partially fed flies can be difficult to distinguish and even those that probe but apparently have not taken a blood meal may become infected.
6. The flies are maintained individually in tubes as described fully in Chapter 3. Sugar meals should be changed daily; oviposition may be discouraged or encouraged by moistening the filter paper, as desired.
7. At the appropriate point the flies are killed and dissected, allowed to re-feed, or processed in some other fashion, depending on the purpose of the experiment. Dissection of infected flies is a skilled procedure that should be practised on uninfected flies first. The fly is first anaesthetized with CO_2 and removed from the tube with a pair of fine forceps. It is transferred to a drop of Locke's solution

on a microscope slide for dissection. The wings and legs are removed using a pair of fine mounted needles and the body transferred to a fresh drop of Locke's solution. Next the head is removed and kept in another drop of Locke's solution. This can be examined later for the presence of metacyclic promastigotes and other forms. At this point promastigotes may emerge into the liquid phase from the severed end of the gut, which is broken at the junction of the midgut and foregut. To remove the gut from the body one needle is placed on the thorax and the other on the tip of the abdomen and gently pulled. The object is to pull the gut out *via* its attachment to the tip of the abdomen. With practice the midgut and hindgut can be removed intact. If difficulty is experienced the eggs can be gently massaged out from a cut made in the abdomen before attempting to remove the gut. Subsequently, the gut can be processed for light microscopy or electron microscopy, or homogenized to obtain parasites for further examination or culture.

10.3.2 Artificial membrane feeding

1. Prepare cage(s) with flies (Section 10.3.1, step 1).
2. Assemble the membrane feeder. The prepared chick skin membrane is laid over the opening of the feeder with the outer surface facing outwards, i.e. ultimately towards the sandflies. The membrane is secured in place by tying around the feeder with cotton thread, then with micropore surgical tape. Obtain assistance as this is difficult to perform alone. Excess skin is trimmed away with a pair of fine dissection scissors. If necessary remove feathers from the membrane: it does not need to be completely naked, but the sandflies should be able to get free access to the surface of the skin. The inlet and outlet tubes are connected to a water supply and the assembly held in a clamp mounted on a retort stand. The water supply is adjusted to the required temperature (Note 5).
3. Prepare the blood and parasite mixture. The final density of parasites in the blood meal can be varied as desired, but commonly densities similar to 2×10^6 per mL are used. It is estimated that an average sandfly blood meal is 0.1 μL, so this gives an inoculum of approximately 200 parasites per fly. Various sources of parasites may be used as indicated in Section 10.2.2, step 2. Although it is possible to supply infected macrophages in the blood meal, isolated amastigotes are more commonly used as these are easier to count in order to prepare a standardized concentration. Relatively crude preparations can be used. For cutaneous parasites the lesion is dissected away from the skin; for visceral parasites the spleen or less commonly the liver, is used. These host tissues are homogenized in a suitable buffer or culture medium to release free amastigotes. They can be partially purified by differential centrifugation: a low speed centrifugation at 200 g for 5 min will remove large debris, host cells and clumps of parasites; a high speed centrifugation at 2000 g for 5 min will pellet the amastigotes (times may need to be extended for large volumes). Cultured parasites are also harvested by centrifugation. Thorough pipetting or passage through a 19 G needle (take care) is needed to resuspend amastigote or promastigote pellets after centrifugation. The parasites can be resuspended directly in the prepared blood. Alternatively they may be resuspended in culture medium first, density checked, then the parasite suspension mixed with the blood, diluting the blood by half (Note 6). The parasite/blood mixture is then introduced into the feeder

using a Pasteur pipette. Before proceeding check that the blood meal does not leak through any undetected holes in the membrane.

4. Place the feeder into the cage. The sleeve to the cage is placed uppermost so that the membrane feeder can be lowered through the opening. Two elastic bands are positioned around the sleeve: these will be used to secure the netting sleeve against the feeder. Again an assistant is useful: one person can lower the feeder whilst the other prevents the sandflies from escaping through the sleeve. The elastic bands are moved into position as the feeder is introduced. The final position of the feeder should be such that it projects into the cage so that it is freely accessible to the sandflies. The membrane feeder is left in position from one to several hours, depending on the experiment and the feeding rate (Note 7).
5. Remove feeder. After a suitable period of time the membrane feeder is removed taking care not to let any flies escape. The parasite/blood mixture should be disposed of safely according to local safety regulations.
6. Tube up flies and count out: Section 10.3.1, step 5.
7. Maintain the flies in tubes: Section 10.3.1, step 6.
8. Dissect or re-feed: Section 10.3.1, step 7.

NOTES

1. More sophisticated systems have been developed that use a peristaltic pump to circulate the blood meal through the feeder (Elkins and Lane, 1987). These can be connected in series, which is especially useful where it is desirable to present the same blood meal simultaneously to different cages of flies.
2. An equally effective membrane can be provided by a hamster pouch (Bastien, 1990).
3. A different strategy is not to introduce the animal into the cage, but to appress the infected animal against the cage; thus allowing the flies to feed through the netting. This does not work with a double-netted cage, a single-netted cage of sufficiently large mesh must be used, and a lesion with a large surface area is required. A further variation is to place flies individually in small glass vials before feeding, then appress the lesion against the netting. Since only one fly can feed at a time, each can be given access for a set period of time.
4. Sandflies are small insects and escapees are hard to detect unless counting is performed at every stage. Check the cage thoroughly for dead flies. Every effort should be made to hunt down and recover any escaped fly. Fortunately, sandflies will not survive for long without the food they require and the likelihood of transmission *via* an escapee is remote, although finite. Insecticides should only be used in the event of a major escape, as their use may render the insectary unusable for a considerable period of time.
5. If poor feeding rates are experienced, this variable should be checked. Optimal temperatures of the feeder normally lies in the range 30–37°C.
6. Membrane feeding has been attempted with a variety of sources of blood, diluted blood, plasma, serum and other concoctions, mainly in specific studies of sandfly feeding physiology. One possible advantage of using diluted blood that is of more general applicability is that this may inhibit egg production and thus prevent oviposition mortality (Ghosh, 1994). In this way it may be possible to obtain longer lasting infections that survive beyond a second blood meal.

7. Unfortunately membrane feeding remains a somewhat unpredictable technique despite all attempts at standardization. Undoubtedly a healthy vigorous sandfly colony is a major contributor to success. Investigators have manipulated all components of the system and environmental factors in efforts to make improvements. However, two practices that are commonly used and are probably useful are to turn the lights out during feeding and to maintain a good temperature differential between the feeder at 30–37°C and the ambient temperature, normally 23–28°C depending on insectary conditions. The age of the flies can also be critical. The longevity of colony-reared flies and the optimal age for feeding depends on the species of fly and may need to be empirically determined (usually 5–10 days).

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CHAPTER ELEVEN

Infection of blackflies with Onchocerca

Albert E. Bianco and Peter J. Ham

11.1 INTRODUCTION

The genus *Onchocerca* comprises approximately 30 species, all of which are obligate parasites of vertebrates and arthropods (Muller, 1979). *Onchocerca volvulus* is the only species to infect humans, the remaining members of the genus being almost exclusively parasites of wild and domesticated ungulates. *Onchocerca* parasites have skin-dwelling first-stage larvae, or microfilariae (mff), that are adapted to transmission by pool-feeding, haematophagous insects with scarifying mouthparts. Consequently, vectors of the various species are insects belonging either to the ceratopogonid midges or to the simuliids, commonly referred to as blackflies on account of the dark coloration of some (but by no means all) species. Blackflies are the vectors of human onchocerciasis (Bianco, 1991).

O. volvulus is an important human pathogen, infecting around 18 million people in subSaharan Africa and Latin America (WHO, 1987). It is the cause of disfiguring subcutaneous nodules (which contain the adult worms), acute and chronic skin lesions (caused by mff) and ocular damage, which may result in partial or complete loss of sight (also caused by mff). In Africa, onchocerciasis is second only to trachoma as the cause of infectious blindness. Owing to the association of infection with rivers that support the aquatic larvae of the *Simulium* vectors, it has acquired the name 'River Blindness' and has been the subject of major control measures designed to break transmission through the application of larval insecticides (Molyneux, 1995).

11.1.1 The need to infect blackflies in the laboratory

There is a great deal of interest in the vector biology of onchocerciasis, prompted by the importance of vector control as a tool in disease intervention. Transmission through much of Africa is maintained by members of the *S. damnosum* species complex, sibling species of which vary in their efficiency as vectors and in their ability to support the development of certain pathogenic or less pathogenic 'forms' of *O. volvulus* (Duke *et al.*, 1966; Duke and Anderson, 1972). It is therefore essential to have methods to measure vectorial competence, involving the controlled infection of blackflies. Moreover, there is a need to generate infective larvae, so as to provide

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material for the development and evaluation of taxonomic probes (e.g. DNA, monoclonal antibodies) which may be utilized as markers for biologically distinct isolates (Unnasch *et al.*, 1989).

Massive financial and logistic support is needed to interrupt transmission of human onchocerciasis by vector control (Molyneux, 1995). There has therefore been much interest in the development of drugs or a prophylactic vaccine for onchocerciasis, which again makes it desirable to have efficient methods for the production of the key life-cycle stages and of experimental infections. Central to these aims is the large-scale production of infective larvae.

11.1.2 Life-cycle in the vector

Following ingestion, mff penetrate the midgut epithelium and migrate through the haemocoele to the thoracic flight muscles. Here they penetrate a muscle fibre and develop intracellularly, growing from approximately 250 μm long × 6 μm wide to 500 μm long × 17 μm wide and moulting twice over a period of 6–8 days. The infective, third-stage larvae (L3) re-enter the haemocoel and migrate to the head and mouthparts to await transmission. As in other nematodes, most postembryonic growth is achieved through increase in cell size and so there is no major change in nuclear content (average of 1.1 ng DNA per larva). Moreover, there is no amplification of parasite numbers in the vector (in contrast to schistosomes and many protozoa), and indeed many of the ingested mff fail to develop. Heavily infected flies may harbour 30 infective larvae or more, although infection is a significant cause of mortality and a major determinant of transmission efficiency.

11.1.3 Principles of laboratory maintenance of *Onchocerca*

It is impractical to attempt the cyclical maintenance of *Onchocerca* parasites in the laboratory because: (i) *O. volvulus* will only develop in the higher apes (humans, gorilla and chimpanzee) (Bianco, 1991); (ii) other members of the genus are host specific to ungulates (Muller, 1979); (iii) the blackfly vectors are extremely difficult to colonize (Section 11.2.4); (iv) the prepatent period is in the order of one year (Bianco, 1991). Therefore, other approaches need to be adopted to provide regular supplies of infected blackflies. In the authors' laboratories, we have tackled this principally by using a local parasite of cattle (*Onchocerca lienalis*) and locally available blackflies (*Simulium ornatum* s.l.). The slaughter of naturally infected cattle provides a source of the skin mff that can be stockpiled in the laboratory by cryopreservation. Blackflies are reared from field-collected larvae and pupae, obviating the need for a sustainable laboratory colony. Highly productive infection procedures, such as intrathoracic injection of mff, are used in place of inconvenient and inefficient methods such as feeding of the vectors on infected hosts. Finally, the infective larvae obtained from blackflies may again be cryopreserved for storage or transport. All of these techniques have also been used by us to produce infective larvae of *O. volvulus*, both within and outside areas endemic for the human disease (Ham and Bianco, 1983b; Bianco *et al.*, 1995).

11.2 MATERIALS

11.2.1 Extraction of *Onchocerca* microfilariae from skin

1. RPMI 1640 medium (ICN Biomedicals).
2. Penicillin and streptomycin (Life Technologies).
3. Heat-inactivated New born Calf Serum (NCS) (Life Technologies).
4. 24 Well plates (ICN Biomedicals).
5. Scalpel, blades, needles, gloves.

11.2.2 Sephadex purification of *Onchocerca* microfilariae

1. PD10 prepacked Sephadex G25 columns (Biotech).
2. RPMI 1640 medium (ICN Biomedicals).

11.2.3 Cryopreservation of microfilariae

1. Ethanediol (ethylene glycol, Sigma-Aldrich).
2. Metal block or plate (preferably aluminium).
3. Glass microscope coverslips.
4. 2 mL Cryotubes (Life Technologies).
5. Liquid nitrogen.
6. RPMI 1640 medium (ICN Biomedicals).
7. Thawing medium (50 mL) is made up as follows:
   20 mL: NCTC 135 tissue culture medium (ICN Biomedicals).
   20 mL: Iscove's Modified Dulbeccos Medium (IMDM) (ICN Biomedicals).
   10 mL: Heat-inactivated fetal calf serum (FCS) (Life Technologies).
   5000 IU/5000 μg: Penicillin and streptomycin (Life Technologies).
8. Cryoprotectant stock solution (×2 strength) is prepared from thawing medium to which is added 18% (v/v) dimethyl sulfoxide (DMSO) (Sigma-Aldrich) and 0.008 M polyvinylpyrrolidone (PVP) (Sigma-Aldrich).

11.2.4 Fly rearing (and colonization)

1. Source of compressed air, e.g. diaphragm pump.
2. Glass or plastic tanks.
3. Airstones and airline.
4. Powdered fish food (e.g. Tetra Ltd).

11.2.5 Infection techniques

(a) Membrane feeding

1. Water bath.
2. Water pump and circulation system.
3. Waterjacketed glass feeding units.
4. Heparinized or citrated bovine blood.

(b) Intrathoracic injection

1. Glass capillary tubes (approximately 10 cm long × 2 mm bore).
2. Micropipettes (Microcaps: Drummond).
3. Tissue culture medium (e.g. TC199/RPMI 1640) (ICN Biomedicals).
4. Fine watchmaker's forceps.
5. Source of carbon dioxide or –20°C freezer.
6. Stereo-dissection microscope.

11.2.6 Maintenance of infected blackflies

1. Humid chamber.
2. Polystyrene pots and mesh (as above).
3. 30% (w/v) dextrose solution containing antibiotics.

11.2.7 Recovery of *Onchocerca* larvae

1. Glass microscope slides.
2. RPMI 1640 medium (ICN Biomedicals).
3. Test sieve (aperture size, 125 μm Endecotts, London UK) (and for aseptic recovery, e.g. for tissue culture).
4. Phosphate buffered saline (PBS).
5. Benzalkonium chloride (17% stock solution, 'Zephiran' Calbiochem).
6. Penicillin and streptomycin (Life Technologies).

11.2.8 Cryopreservation of infective larvae

1. NCTC 135 tissue culture medium (ICN Biomedicals).
2. Iscove's Modified Dulbeccos Medium (IMDM) (ICN Biomedicals).
3. Heat-inactivated FCS (Life Technologies).
4. Penicillin and streptomycin (Life Technologies).
5. Dimethyl sulfoxide (DMSO) (Sigma–Aldrich).
6. Polyvinylpyrrolidone (PVP) (Sigma–Aldrich).
7. 2 mL Cryotubes (Life Technologies).

11.3 METHODS

11.3.1 Extraction of *Onchocerca* microfilariae (mff) from skin

Large numbers of *Onchocerca* mff of value for a range of experimental purposes can be obtained from the skin of cattle harbouring natural infections of *O. lienalis* (Bianco *et al.*, 1980). The parasite has a wide geographic distribution, and so should be available to many investigators. Skin samples of approximately 20 cm in diameter are collected from the animals at slaughter from around the umbilicus. It is usually necessary to collect samples from around 30–60 mature animals (i.e. dairy rather than beef cattle) to be reasonably sure that some will contain worthwhile numbers of mff.

(a) To test skin for the presence of mff

1. Shave a small patch of hair from a spot approximately 5 cm from the centre of the umbilicus along the mid-ventral line.
2. Using a needle, lift a cone of skin and cut it off with a scalpel. The 'skin snip' should be roughly 0.5–1 cm across.
3. Place the biopsy in the well of a Linbro plate loaded with 1–2 mL RPMI 1640.
4. Incubate at 37°C for 60 min.
5. Remove the biopsy and allow the plate to stand for 5 min (for mff to sediment). View under highest magnification of dissecting microscope using transmitted light.

(b) To extract mff in large numbers

1. Wash infected skin samples and shave thoroughly with a large convex scalpel blade. Only use samples with the highest densities of mff.
2. Pin the skin to the cork board epidermis up, using 21 or 18 G needles. The skin should be stretched as taut as possible.
3. Spray the surface with 70% ethanol, and quickly remove excess with a clean tissue. Repeat.
4. Leave skin to air-dry 10 min.
5. Observing aspectic technique, use a needle and scalpel to remove long slivers of skin from the entire surface of the sample. These should be almost as deep as the dermis, but should not go through to the subcutaneous connective tissue (which is highly contaminated). Avoid the edge of the sample for the same reasons.
6. Place the slivers of skin directly into 50 mL tubes or 30 mL universal containers half-filled with incubation medium (RPMI with 200 IU penicillin and 200 μg streptomycin per mL plus 20% serum). Do not pack the skin tightly.
7. Incubate at 37°C for 4 h or overnight at RT. (Note: pH will be low after overnight incubation.)
8. Shake tubes vigorously to dislodge mff stuck to the skin slivers and decant the medium.
9. Centrifuge the medium at 600 g fpr 15 min at RT.
10. Remove the supernatant carefully and resuspend the mff in 10 mL fresh RPMI plus 20% NCS prewarmed to 37°C. Leave 15–60 min to allow mff to revive (they may be sluggish if coming out of acidic medium).
11. Filter the mff through a pre-soaked sponge filter held in the barrel of a 5 mL syringe. Make sure the filter allows free passage of liquid through it. If not, use another filter or the mff may be lost.
12. Count the mff in five 10 μL aliquots. Make up to the desired concentration by centrifugation and resuspension as in step 9.
13. Use mff directly or purify further on a PD-10 column (protocol below).

11.3.2 Sephadex purification of *Onchocerca* microfilariae

The recovery of mff from skin under aseptic conditions may be difficult to achieve under some circumstances, resulting in contamination with bacteria or yeasts. These can be extremely detrimental to blackflies when using the intrathoracic infection techniques for the production of infective larvae. If the batches of mff are not too severely contaminated, these can be purified by gel filtration: microbial contaminants

in the suspension pass through the Sephadex column ahead of the mff (Taylor *et al.*, 1984).

1. Pre-equilibrate PD10 column with 25 mL warm medium.
2. Load mff onto column suspended in 1–2 mL (max) medium. Allow to run in. Preparations should not be excessively turbid and should be diluted and run on more than one column if necessary.
3. Run in a further 2 mL medium and then start to wash column with a continuous flow of medium.
4. Collect 2 mL fractions.
5. Expect contaminating microbial pathogens and host cells to elute first. *O. lienalis* mff elute in approximately 6–20 mLs.
6. Inspect each fraction and pool mff from cell-free fractions. If there is excessive cell contamination the fractions can be re-run on the original column (or a new one if this is clogged).

11.3.3 Cryopreservation of microfilariae

These are extremely useful and reliable methods for storing and transporting mff. Importantly, they are easy to use in the field and require no sophisticated controlled-cooling apparatus. Only a minor modification to the original ethanediol method developed for *Onchocerca* mff was required to adapt it to *Brugia* (Ham *et al.*, 1981; Ham and Bianco, 1981; Ham and Townson, 1986).

(a) Ethanediol procedure for isolated microfilariae

1. Estimate total number of mff and concentrate by centrifugation at 600 **g** (1650 rpm) for 15 min. Draw off supernatant with care so as not to disturb mff pellet.
2. Resuspend mff thoroughly but gently in 50–100 μL serum-free tissue culture medium (RPMI 1640, 199 medium, etc.) up to a concentration of 2×10^6 mff/mL. If the mff are left too long after centrifugation (step 1) the pellet will not disperse easily and clumps of mff will remain that tend to be lost during pipetting.
3. Measure the volume of mff suspension using a graduated pipette.
4. Add ethanediol (i.e. ethylene glycol) slowly and with mixing to a final concentration of 10% (v/v) (i.e. divide vol of parasites by 9 and add that amount of ethanediol).
5. Incubate parasites at 37°C for 12–15 (max) min.
6. Meanwhile, put the metal plate onto a bed of ice to cool. Pre-cool the 70% ethanediol solution in the ice bath.
7. Transfer mff suspension from 37°C to the ice bath after step 5.
8. Wipe condensation off the metal plate and lay 2 or 3 glass strips on top, overhanging the plate at one edge (to permit the strips to be gripped with forceps).
9. Pipette 10 μL of 70% ethanediol on one end of each glass strip.
10. Change tip and mix contents of tube containing mff by swirling. Take 10 μL of mff suspension and add to the ethanediol on the glass strip while mixing and spreading the liquid into a thin layer for 15 s exactly.
11. With the forceps, slide the coverslip off the metal plate and snap freeze it by waving the slip vigorously under liquid nitrogen. The medium will not drip off the coverslip if this action is carried out smoothly but decisively. Hold it under

the liquid nitrogen until it stops hissing and transfer it to a Nunc tube already filled with liquid nitrogen (the strips must now be kept under nitrogen at all times). Drops frozen correctly will have a glassy appearance (not opaque).

12. Repeat procedure until all mff suspension is used up.
13. Calculate number of mff per coverslip by dividing original number of parasites (step 1) by the number of slips. In practice, the numbers per slip often vary if the mff suspension is not kept uniform during pipetting.
14. To thaw, prewarm a solution of RPMI 1640 medium containing 20% heat inactivated NCS or FCS to 37°C in a water bath or dri-block (an incubator is no good).
15. To 2 mL of prewarmed medium in a prewarmed tube add one glass slip covered with frozen mff. The glass slip must be thawed rapidly by taking it straight from the liquid nitrogen and plunging it directly into the prewarmed medium in one quick action. Mix rapidly and return to the water bath for 5–10 min.
16. Remove glass slip from medium and check mff motility (should be >90%). A few mff may be damaged (e.g. broken). With this method around 75% of the mff are viable as compared with unfrozen parasites based on their ability to develop to L3 stages in the vector.

(b) Methanol procedure for microfilariae in skin biopsies

Often mff are available with little time available to isolate them from the skin snips. This technique was developed to provide successful preservation of parasites within the biopsies. It is rapid and simple, requiring little equipment.

1. Place positive skin biopsies into preprepared TC199 or RPMI1640 containing 20% (v/v) methanol at –20°C.
2. Incubate the snips for 10 min at –20°C.
3. Remove the snips and surface dry on filter paper.
4. Drop the snips individually into liquid nitrogen.
5. The snips can be placed into a Nunc cryotube and stored indefinitely in liquid nitrogen.
6. To thaw, remove a snip from liquid nitrogen using forceps and quickly place into 5 mL of medium at 37°C. The transfer needs to be rapid to prevent slow thawing which damages the parasites within the tissue.
7. Allow the snip to incubate for 1–2 h as normal. The viable mff will migrate out of the tissue into the medium and can be collected for use.

11.3.4 Blackfly maintenance and colonization

Blackflies have aquatic larval and pupae stages. Adult females lay eggs in fast flowing rivers and streams. These hatch and the larvae attach to substrates such as rocks and vegetation. A fastidious requirement for high oxygen levels, flowing water and fine particulate food make this a difficult group of insects to rear in the laboratory. In addition, adult insects rarely mate in such conditions and only a few successful reports of simuliid colonization occur (Simmons and Edman, 1982; Brenner *et al.*, 1981; Ham and Bianco, 1984). For practical purposes we shall confine this procedure to that of rearing wild collected larvae and pupae to adults in the laboratory. Chapter 4 of this text, however, is devoted to the specific methods involved in colony maintenance of blackflies and should be consulted for more details on this subject.

1. Substrates such as stones and vegetation bearing larvae and pupae are collected from breeding sites in rivers.
2. Place the material in large, well aerated tanks of water (approximately 150 L).
3. Cover the tanks and place mesh cages over holes in the cover.
4. Adult flies emerging from the pupae will be attracted up onto the cages using overhead illumination. The use of funnel traps made from powder funnels, prevents the insects flying back down to the water and drowning.
5. Remove the blackflies at regular intervals and place into maintenance pots described below (Section 11.3.6).
6. Once larvae pupate, a period of around 3 days is required before eclosion or adult emergence. Although pupae do not feed, the larvae must be provided with powdered tropical fish food (e.g. Tetra Ltd), to allow development and successful pupation and subsequent emergence.

11.3.5 Infection techniques

There are three basic approaches, of which the most logical (natural feeding) is the most difficult and least efficient for most *Onchocerca* spp. An important exception is *O. volvulus*, which may be effectively produced by permitting blackflies to feed on human volunteers (Cupp *et al.*, 1988). This will, nevertheless, be impractical for all but a handful of laboratories based in endemic areas and has therefore not been included as a specific protocol. Readers are referred to the published methods of Cupp *et al.* (1988) and are reminded that such procedures will require appropriate ethical clearance.

(a) Membrane feeding

This procedure can be used for experimental infection of several species of blackflies, either laboratory reared or wild caught as adults (Ham and Banya, 1984). Feeding on blood is performed through a membrane attached to a water jacketed unit in which the blood is kept.

1. Blackflies must be prepared prior to feeding by placing them in the cage to be used (normally small). Sugar food can be withdrawn for 12 h before blood-feeding and sometimes witholding water for a few hours can assist in obtaining a good blood meal, although care needs to be taken to prevent desiccation. Maintaining the insects in the dark is also believed to help induce subsequent feeding.
2. Cover the base of the feeding unit with 1-day-old chick skin, or other artificial membranes such as sausage skin. The skin can be held in place with an elastic band and should be fitted such that the outer side of the skin will face the mesh covered top of the cage containing the blackflies.
3. Introduce fresh or frozen/thawed blood containing mff at the desired concentration (20 000–60 000/mL) into the top of the feeding unit, taking care to prevent introduction of air bubbles.
4. Switch on the supply of warmed circulating water set at approximately 38–39°C.
5. Check for blood leaks. When the blood has reached the required temperature, remove the cage of blackflies from the dark and place under the feeding unit, so that it is on top of the mesh covered cage.
6. Overhead illumination can assist in attracting flies onto the membrane.

7. Ensure that the blood is topped up and kept agitated throughout the feed, pipetting the blood up and down gently. This will prevent mff from clumping and ensures an even infection of all fed flies.
8. Individual insects will only require 3–5 min to feed to repletion. However, a cage of insects should be allowed access to the blood until no further feeding can be seen.
9. Following the blood meal, the replete blackflies should be removed and housed in pots as in Section 11.3.6.

(b) Intrathoracic injection

This method of infection allows a high infection rate, and a high intensity of infection among susceptible blackflies (Ham and Bianco, 1983a; Bianco *et al.*, 1989). Counted doses of mff can be given individually using fine extruded glass capillary needles to inoculate the parasites directly into the thorax of the simuliids, where they develop. This method has proved extremely productive and practical, yielding consistently good numbers of infective larvae, when required, in a predictable manner.

1. Prepare finely extruded needles by drawing the glass capillaries over a flame.
2. Prepare the blackflies by anaesthetizing a small number at a time using carbon dioxide. If this is not available they can be immobilized by placing them into a freezer at –20°C for a few minutes.

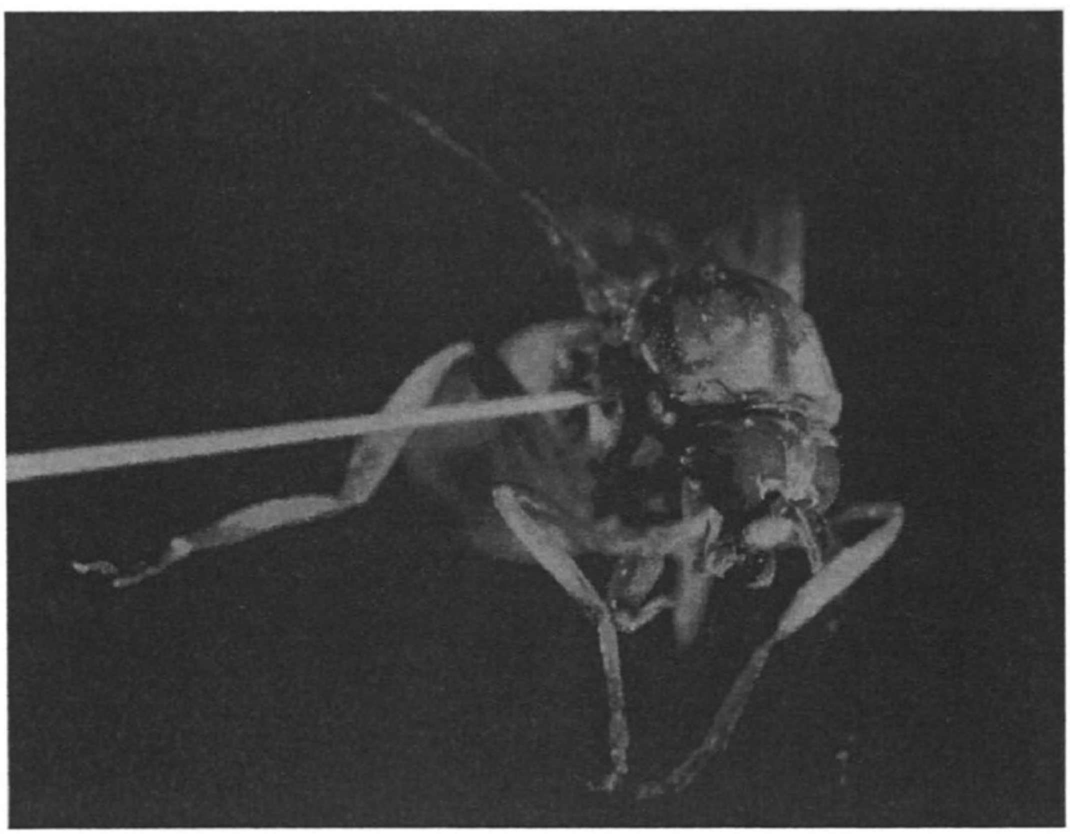


Figure 11.1 Blackfly receiving microfilariae by intrathoracic injection. Note the relative sizes of the insect and injection needle. The needle tip is inserted through the pleural membrane, ventral to the wing base. A typical inoculum volume is around 1 μL.

3. With the aid of a stereo microscope, load counted doses of mff up into a series of needles, such that the required number are in no more than 1–2 μL of tissue culture medium. The tips marketed as 'Microcaps' by Drummond are suitable for taking up and expelling the fluid from the needles (Figure 11.1).
4. Again, while observing under a stereo microscope, insert the needle laterally into the thorax of the anaesthetized blackfly, taking care not to inject dorsally or ventrally. Inoculation *via* the thin pleural membrane at the base of the wing is a preferred site, resulting in little or no mortality of the flies. This procedure is assisted by using a pair of fine forceps as a support for the insect.
5. Carefully expel the fluid into the insect. A slight increase in abdominal volume may be seen. Also take care not to allow fluid to leak out of the site of inoculation. Remove the needle.
6. Inoculated insects should be maintained as described in Section 11.3.6.

(c) Semi-automated injection

This method employs a device that uses pulses of compressed air to discharge precise volumes of inoculum from an injection needle containing a high density suspension of mff (Bianco *et al.*, 1989). The technique is more efficient than the individual pipette method, but does require the construction of specialized instrumentation (described in the publication). It also assumes that access to large numbers of mff is not a limiting factor. Examples of infective larvae production in blackflies infected by manual and semi-automated techniques are given in Tables 11.1 and 11.2.

Table 11.1 Production of *Onchocerca lienalis* infective larvae (L3) in *Simulium ornatum sl.* by means of individual micropipettes for the injection of microfilariae. The data shown are representative of the technique in routine use

Fly batch	*No. flies infected*	*No. surviving on day 7 (%)*	*Mean recovery of L3/survivor*	*Total L3* recoveries*
1	290	148(51%)	13.9	2052
2	150	96(64%)	25.2	2423
3	131	45(34%)	38.6	1739
4	175	82(47%)	18.4	1507
5	200	148(74%)	13.1	1932
6	400	267(67%)	17.8	4742
7	600	174(29%)	16.4	2854
8	150	50(33%)	18.5	927
9	500	340(68%)	21.0	7131
10	150	72(48%)	23.3	1681
11	189	116(61%)	17.0	1977
12	300	190(63%)	19.5	3698
13	200	149(75%)	28.2	4203
Overall	3435	1877(55%)	19.6	36,866

* The recoveries given are for infective larvae from the head and mouthparts only. Total yields of third stage larvae will be approximately 20% greater.

Table 11.2 Production of *Onchocerca lienalis* infective larvae (L3) in *Simulium ornatum sl.* by means of a semi-automated system for the injection of microfilariae. The data shown are derived from routine laboratory records

Fly batch	*No. flies infected*	*No. surviving on day 7 (%)*	*Mean recovery of L3/survivor*	*Total L3* recoveries*
1	572	220(38%)	10.6	2324
2	508	128(25%)	21.8	2784
3	250	140(56%)	18.1	2536
4	525	126(24%)	10.2	1286
5	177	87(49%)	10.3	896
6	1000	202(20%)	16.8	3403
7	1125	420(37%)	13.2	5539
8	630	345(55%)	12.5	4319
9	281	206(73%)	14.7	3019
10	195	126(65%)	14.0	1770
11†	3000	680(23%)	20.8	14112
12	600	281(47%)	26.7	7498
13	500	244(49%)	13.6	3320
14	350	198(57%)	10.1	2000
15†	4700	1053(22%)	14.3	15087
Overall	14 413	4456(31%)	15.7	69 893

* The recoveries given are for infective larvae from the head and mouthparts only. Total yields of third stage larvae will be approximately 20% greater.
† Not all flies infected on the same day, but dissected as a batch.

(d) Use of nonsimuliid surrogate vectors

1. Alternatives to simuliids have been assessed as possible laboratory vectors of *Onchocerca* infections. In particular, mosquitoes, including *Aedes aegypti*, have been used (Bianco and El Sinnary, 1980; Lok *et al.*, 1980). However, the yield obtained from these insects has not been high.
2. Nonvector species of blackflies have also been used as surrogates for bovine and human *Onchocerca* infections with considerable success (Ham and Bianco, 1983a, 1983b; McCall and Trees, 1989).

11.3.6 Maintenance of infected blackflies

Once infected, it is necessary to maintain the simuliids in an undisturbed environment in order to attain high survival rates (Bianco *et al.*, 1989).

1. Place blackflies in small polystyrene pots (approximately 15 × 15 cm) covered in suitable nylon or cotton mesh.
2. Feed infected insects on 30% (w/v) dextrose solution containing antibiotics and fungicide (200 U penicillin, 200 μg streptomycin, 100 U nystatin/mL sugar), administered *via* cotton wool pads placed on the cages.

3. Place the pots containing the simuliids into a humid chamber (approximately 85% RH) in the dark. Change the sugar pads at regular intervals to prevent fungal growth.
4. The development of microfilariae to infective larvae is dependent on temperature and the species of insect and parasite being used. A general guide is that bovine *Onchocerca* species take approximately 7 days at a temperature of 27°C. An increase or a decrease in temperature, by one degree, effectively decreases or increases this time respectively by one day.

11.3.7 Recovery of *Onchocerca* larvae

This section is divided into three related procedures: the recovery of developing larvae from individual insects, mass recovery of infective-stage larvae, and the collection of larvae under aseptic conditions as required for subsequent *in vitro* cultivation (Bianco *et al.*, 1989; Lok *et al.*, 1984).

(a) Recovery of larvae from individual flies

1. Dissect blackfly with mounted needles or watchmaker's forceps into head, thorax and abdomen in individual drops of RPMI medium on a microscope slide.
2. Tease the tissues apart and add a second drop of medium with care, ensuring the drops do not spread. Do not add a coverslip at first; you may wish to recover the worms which are easily damaged by pressure or shearing forces.
3. Look for larvae under higher magnifications with transmitted light. Phase or Nomarski differential interference contrast are the best forms of illumination. First- and second-stage larvae will occur exclusively in the thorax, whilst third-stage larvae may arise throughout the body, concentrating in the mouthparts and head. In heavy infections, infective larvae may even spill over into the halteres and legs.

(b) Mass recovery of infective larvae

1. Dissect two or three insects by the method above, to check that they contain infective larvae at the appropriate stage of development.
2. Knock down the remaining flies with CO_2 or by placing them at –20°C for 30–60 s (don't let them freeze!).
3. Place the flies in a small pool of RPMI medium on a large glass plate (approximately 10–20 cm square).
4. Crush the flies by gentle rolling under a glass test tube, exerting sufficient pressure to cause the rupturing of their heads.
5. Flush the flies and medium into a Baermann apparatus, comprising a 50 mL funnel with wire test sieve (aperture 125 μm), filled with RPMI medium prewarmed to 37°C. Leave for 90 min at 27°C.
6. Tap off medium into a Petri dish and collect larvae by pipette under a low-power stereo microscope.

(c) Recovery of larvae under aseptic conditions for in vitro *culture*

1. Knock down flies with CO_2 or by placing them in the cold.
2. Drop flies into PBS containing 1% Zephiran (i.e. 3 mL of 17% stock in 50 mL) to surface sterilize the insects.

3. Swirl for 60 s and pour off fluid through a sieve (to prevent loss of flies).
4. Quickly rinse three times with PBS leaving in the third change for 1 min.
5. Wash for 5 min in PBS with 1000 IU penicillin/1000 μg streptomycin/mL. Repeat once. Then continue the procedure in a laminar flow hood.
6. Remove heads and place in single drops of RPMI with 500 IU penicillin/500 μg streptomycin/mL in a sterile Petri dish. Do not tease the tissues. Place in humid box at 37°C for 30–60 min.
7. Collect larvae with a finely extruded sterile Pasteur pipette and load into a 15 mL conical tube.
8. Wash larvae three times by centrifugation (200 g for 5 min) and resuspension in medium without antibiotics (10 mL per change). Ensure supernatant medium is parasite-free before discarding (i.e. examine spent medium in a Petri dish under a dissecting microscope).

11.3.8 Cryopreservation of infective larvae

There are a number of methods described for the cryopreservation of filarial larvae. Some are based on the low temperature storage of larvae within flies, and others on the freezing of larvae in medium following their recovery from insect tissues. The latter is more convenient (one can calculate how many parasites one wishes to thaw) and, by and large, gives better results. The method described here was originally developed by Lowrie for *Brugia* and *Acanthocheilonema* third-stage larvae (Lowrie, 1983), and appears to adapt well to *Onchocerca* parasites (J.B. Lok pers. comm.; authors' unpublished observations).

1. Make up counted batches of third-stage larvae in thawing medium by centrifugation (100 g × 5 min) and resuspension. Concentrate each batch into 0.5 mL medium and transfer to a 2 mL Nunc cryotube.
2. Add 0.5 mL of cryoprotectant stock solution to each tube and mix gently.
3. Cool at –0.8 to –1.0°C per min to –70°C, using either: (i) a controlled cooling apparatus; (ii) 'freezing tray' (Taylor Wharton, UK) inserted into the neck of a liquid nitrogen container (Taylor Wharton 35HC); or (iii) a polystyrene box containing a rack supported above a freezing mix of methanol and dry ice. The freezing rate should be calibrated in advance to fall within the requisite range.
4. Plunge tubes in liquid nitrogen and store at –196°C.
5. To thaw, transfer tubes directly from liquid nitrogen into a waterbath prewarmed to 37°C. Rapid thawing is important, although the contents should not be agitated during defrosting (which can result in damage to larvae).
6. Transfer contents to a 15 mL tube and top-up with pre-warmed thawing medium.
7. Centrifuge at 100 g for 5 min and discard supernatant medium. Refill tube and repeat once.
8. The larvae will slowly regain activity over a number of hours.

NOTES

Most of the important technical features of these methods are given under the relevant heading. Common difficulties encountered by those new to the methodology are the following:

1. Clumping of microfilariae during filtration and uptake into injection needles: Rectified by resuspension.
2. Microbial contamination of parasite suspension: Rectified by improved aseptic technique, short incubation of the skin in medium, or passage of suspension through PD-10 column.
3. Poor blood-feeding rates during infection by membrane feeding: Rectified by starvation and maintenance in the dark prior to feeding.
4. High mortality following intrathoracic injection: Rectified by attention to each of the following: (i) use of fine needles; (ii) minimizing inoculum volume; (iii) reducing exposure time to carbon dioxide; (iv) reducing number of parasites per inoculum; (v) use of antibiotics in the sugar solution; (vi) reduction of fly density in cages; (vii) poor injection technique. A useful way of monitoring problems of mortality after injection is to set up controls: These should include untreated flies and sham-operated insects (i.e. injected with parasite free medium).

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CHAPTER TWELVE

Infection of tsetse with Trypanosoma brucei rhodesiense *and* T. b. gambiense

Ian Maudlin

12.1 INTRODUCTION

Trypanosoma brucei rhodesiense and *T. b. gambiense* are salivarian trypanosomes (subgenus *Trypanozoon*) which are major pathogens of humans causing, respectively, the acute (Rhodesian) and chronic (Gambian) forms of sleeping sickness in Africa. Both trypanosome species are transmitted during feeding via the salivary glands of tsetse flies, both sexes of which are exclusively haematophagous. Trypanosomes must overcome two obstacles in completing their life-cycle in the fly: (i) they must establish in the midgut and (ii) mature from midgut to mammalian infective forms in the salivary glands.

12.1.1 Establishment of trypanosome infections in the midgut of tsetse

In the mammalian host the entire surface of African trypanosomes is covered in variant surface glycoprotein (VSG) which protects against the mammalian immune system. On entering the tsetse midgut the parasite faces very different environmental conditions: a drop in temperature from 37°C to approximately 25°C, a battery of digestive enzymes and a much more primitive, nonspecific, 'immune' system. As glucose disappears rapidly from the blood meal (within 15 min of ingestion) bloodstream form trypanosomes must quickly develop the ability to use alternative substrates – they quickly differentiate to the procyclic or insect form (accompanied by ultrastructural and biochemical changes). The bloodstream form trypanosome also sheds the VSG coat replacing this with an invariant coat of procyclins (or PARPs).

Transformation to the procyclic form can be induced *in vitro* within 48 h by dropping the temperature of bloodstream forms to 25°C. For an individual cell, the VSG coat is lost in about 4 h while it takes 12–16 h to express a maximum level of procyclin at the surface. Transformation to the procyclic form takes place much more rapidly in the tsetse midgut than *in vitro*; Turner *et al.* (1988) found that half of bloodstream form trypanosomes in the posterior midgut (PMG) lose their VSG coat within 2–3 h of ingestion. Proteases, largely trypsin, are secreted in the PMG and it has

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been suggested that a midgut protease acts as the trigger for differentiation from bloodstream to procyclic forms (Imbuga *et al.*, 1992; Osir *et al.*, 1995). It may be that proteases speed up the process but as differentiation takes place quite rapidly in the absence of such stimuli *in vitro* simply by dropping the temperature of cultures, it is doubtful that proteases are essential triggers for transformation.

Wild tsetse populations are highly resistant to the establishment of midgut infections and many factors have been shown to influence establishment (for review Maudlin, 1991), the most obvious being tenerality; flies which have not taken a blood meal are far more susceptible to infection than flies which have previously been fed (Welburn and Maudlin, 1992). Most bloodstream form trypanosomes entering the fly midgut fail to transform and simply die (Turner *et al.*, 1988), but it takes flies about 5 days to completely clear trypanosomes from their midguts (Welburn *et al.*, 1989). A single surviving trypanosome is however all that is needed to establish a midgut infection (Maudlin and Welburn, 1989).

The central role of midgut lectins in refractoriness of tsetse to infection was shown by feeding inhibitory sugars (D+glucosamine and GlcNac) with the infective feeds; this had a dramatic effect on infection rates in the midgut with >90% teneral *Glossina mortisans morsitans* establishing midgut infections (Maudlin and Welburn, 1987). This suggested that the normal action of the lectin *in vivo* was to prevent colonization of the fly gut by parasites. Mihok *et al.* (1992) have since confirmed the *in vivo* action of D+glucosamine in *G. m. centralis* and *G. pallidipes*.

A blood meal induced lectin (60 kDa), the activity of which is strongly inhibited by glucosamine, has been isolated from *G. longipennis* (Osir *et al.*, 1995; Abubakar *et al.*, 1995). Feeding purified PARP with the infective feed significantly increases midgut infection rates in tsetse (Welburn and Pearson, unpublished observation) suggesting that trypanosome death normally results from midgut lectin binding to PARP on the procyclic surface.

Selection experiments showed that susceptibility to midgut infection in tsetse was a maternally inherited character (Maudlin, 1982) related to the inheritance of symbiotic rickettsia-like organisms (RLO). The relationship between RLO and susceptibility to infection was shown to be quantitative (Welburn and Maudlin, 1991). It was suggested that GlcNAc, which would specifically inhibit the potentially lethal midgut lectin, would be produced by the chitinolytic action of the fly's symbiotic bacteria on chitin (Welburn *et al.*, 1993). RLO numbers increase during larval/pupal development in tsetse (Welburn *loc. cit.*) and the greater susceptibility of the teneral fly may be due to the action of RLO on chitin during pupation.

12.1.2 Maturation of trypanosome infections in the tsetse

It is important to realize that only a proportion of flies with midgut infections develop mature infections; several factors influence this transmissibility including fly species, fly sex and trypanosome genotype (Maudlin and Welburn, 1994). Fly species greatly affects transmissibility: *G. m. morsitans* will mature all midgut infection of some *T. congolense* stocks (Welburn and Maudlin, 1989) whereas *G. p. palpalis* rarely produces a mature infection with the same stocks (Welburn *et al.*, 1994). Males promote greater transmissibility than females suggesting the operation of a sex-linked recessive gene (Maudlin *et al.*, 1991) but further analysis revealed variation in estimates of gene frequency suggesting a sex-limited rather than a sex-linked gene (Dale *et al.*, 1995).

12.1.3 Infection of tsetse in the laboratory with *T. b. rhodesiense*

The use of mammalian blood to infect tsetse flies, whether using the host directly or presenting the infected blood under an artificial membrane, creates problems when experimental conditions need to be replicated. Salivarian trypanosome infections in mammals characteristically show an undulating parasitaemia which makes it difficult to standardize the infective dose. Moreover, *Trypanosoma brucei* s.l. are pleomorphic in the blood; long, slender, stumpy forms being predominant during remission; and this pleiomorphism is critical to infectivity, as the stumpy forms are responsible for initiating infection in the fly (Vickerman and Barry, 1982).

Feeding culture-form procyclic trypanosomes to flies through a membrane (Evans, 1979) may also present problems of repeatability, as the infection rate can be dependent on the diet used in the infective feed (Gingrich *et al.*, 1982; Maudlin *et al.*, 1984). Schoni *et al.* (1982) showed that *in vitro* cultured bloodstream form *T. b. brucei* could be used to infect flies, but we have found that this type of culture results in much larger variation in fly infection rates between experiments than when infected mice are used (Maudlin *et al.*, 1986). In order to standardize the infective feed we have developed a method which uses frozen infected mouse blood-fed to flies through an artificial membrane.

This method of infection was compared with the use of fresh infected mouse blood using a single trypanosome stock. Three groups of *G. m. morsitans* were used, two of which were infected with stabilates of frozen mouse blood each suspended in 4 mL of fetal calf serum with 0.5 mL washed pig red cells (Groups 1 and 2 – infective doses 2×10^6 and 5×10^5 trypanosomes per mL respectively). The third group of flies (Control) was infected with 0.25 mL of fresh infected mouse blood suspended in the same fetal calf serum/pig red cell mixture to give an infective dose of 2×10^6 trypanosomes per mL. Flies failing to feed were removed from the experiment. The flies were maintained on an *in vitro* diet of defibrinated pig blood and dissected 21 days post-infection. Results showed no significant heterogeneity in infection rates between flies fed on fresh infected mouse blood and those fed on frozen samples. We have now adopted this technique for routine use in the laboratory, and we have obtained consistent infection rates with *T. b. rhodesiense* stocks. This method of fly infection has the advantage that the infective dose, trypanosome stock and parasitaemic state of bloodstream form trypanosomes can be replicated many times. For example, a group of ten infected mice with a parasitaemia in the range 1.2×10^8 to 1×10^9 per mL will yield enough frozen material, when diluted, to provide at least 50 frozen aliquots. In practice each aliquot when reconstituted and diluted in 5 mL of blood will yield a final dose of 1×10^6 to 4×10^6 trypanosomes per mL in the infective feed. One 5 mL infective feed is sufficient to infect 50 teneral flies. Given the recent interest in trypanosome hybridization in the tsetse fly (Jenni *et al.*, 1986), this method should be particularly useful for the presentation of mixed infections to flies. The numbers of experimental animals used for fly infection experiments may also be greatly reduced.

12.1.4 Infection of tsetse in the laboratory with *T. b. gambiense*

T. b.gambiense presents a special case for infection of tsetse in that all laboratories engaged in this work have found this trypanosome the most difficult to deal with; midgut infections are obtained only with difficulty and salivary gland infections are a rarity.

The first problem with *T. b. gambiense* is to obtain bloodstream forms for experimental purposes. The infection of mice with *T. b. gambiense*, even when immunosuppressed, is difficult and the use of *Mastomys* rats is recommended by some authorities. Reasonable midgut infections can be obtained with *T. b. gambiense* if D-glucosamine is used in the infective feed (Dukes *et al.*, 1989).

12.2 MATERIALS

12.2.1 Procurement of tsetse flies

1. Tsetse flies for experimental studies can be obtained through the following sources: (i) Dr Udo Feldmann, Insect and Pest Control Division, IAEA, Wagrammerstrasse 5, PO Box 100, A-1400, Vienna, Austria, or (ii) Dr I Maudlin, Anderson College, Division of Molecular Genetics, University of Glasgow, G12 8QQ.
2. Tsetse will be despatched as pupae and should be stored at 25°C in damp sand.
3. Flies will emerge approximately 30 days after the date of deposition. On emergence tsetse flies should be placed in cages and maintained at 25°C and approximately 70% RH (*G. m. morsitans*, *G. pallidipes*) or 90% RH (*G. p. palpalis*).

12.2.2 *In vitro* cultivation and cryopreservation of trypanosomes

1. Cunningham's medium: African trypanosomes are maintained in Cunningham's medium, a complex medium, best prepared in bulk then aliquoted. Contains the following ingredients (for 4 L):
   19.080 g HEPES
   2.120 g NaH_2PO_4
   12.160 g $MgCl_2.6H_2O$
   14.8 g $MgSO_4.7H_2O$
   5.92 g KCl anhydrous
   0.6 g $CaCl_2.2H_2O$
   2.8 g Glucose
   1.6 g Fructose
   1.6 g Sucrose
   2.68 g L-Malic acid
   1.48 g Gamma-ketoglutaric acid
   0.22 g Fumaric acid
   0.240 g Succinic acid
   8.0 g Beta-alanine
   4.36 g DL-alanine
   1.76 g L-Arginine
   0.96 g L-Asparagine
   0.44 g L-Aspartic acid
   0.32 g L-Cysteine HCl
   0.120 g L-Cysteine
   1.00 g L-Glutamic acid
   6.560 g L-Glutamine

0.480 g Glycine
0.640 g L-Hystidine
0.36 g DL-Isoleucine
0.36 g L-Leucine
0.6 g L-Lysine
0.8 g DL-Methionine
0.8 g L-Phenylalanine
27.7 g L-Proline
0.8 g DL-Serine
1.08 g L-Taurine
0.4 g DL-Threonine
0.4 g L-Tryptophan
0.8 g L-Tyrosine
0.84 g DL-Valine
8.0 mL Vitamin mixture
(BME ×100)
Phenol red (0.5% w/v)
Double distilled water (ddH_2O)

2. Gentamicin stock solution: 50 mg/mL (Gibco BRL, UK)
3. Phosphate-buffered saline-glucose (PSG) 6:4 for cryopreservation of bloodstream form trypanosomes (Lanham and Godfrey, 1970). Add the following reagents to a final volume of 1 L ddH_2O: 8.09 g Na_2HPO_4, 0.47 g $NaH_2PO_4.2H_2O$, 14.95 g Glucose, and 2.63 g NaCl, mix, and filter sterilize.
4. Fetal calf serum.
5. Heparin: commercially available.
6. Cyclophosphamide stock solution: 500 mg powder ('Endoxana', ASTA Pharma AG, Frankfurt) dissolved in 25 mL ddH_2O.
7. Halothane: commercially available.
8. 1 mL Cryotubes (Nunc, Denmark).
9. Freezer at –70°C and liquid N_2 containers.

12.2.3 Fly feeding using *in vitro* technique

1. Sterile blood (e.g. horse blood obtained by venous puncture) can be obtained commercially (Tissue Culture Services, UK).
2. Silicone membranes – these may be prepared as described (Bauer and Wetzel, 1976).

12.3 METHODS

12.3.1 Preparation of Cunningham's medium

1. The solid ingredients listed in the materials section are dissolved in ddH_2O as follows: all inorganic salts except $CaCl_2$, in 400 mL, $CaCl_2$ in 200 mL, sugars in 400 mL, organic acids in 200 mL, amino acids in 2600 mL.
2. Mix solutions and adjust pH to 7.4 using 2 N NaOH.

3. Sterilize by filtration through Millipore 0.22 μm filter. Store at –20°C. Thaw before use and add fetal bovine serum (either non-inactivated or heat-inactivated) to 20% v/v.

12.3.2 Preparation and cryopreservation of bloodstream form trypanosomes

1. Mice should be immunosuppressed with 0.1 mL cyclophosphamide 24 h before infection with trypanosomes.
2. Parasitaemia is monitored by examination of tail blood.
3. When peak parasitaemia is reached (strain-dependent) mice are killed with halothane and exsanguinated from the heart using heparinized syringes containing 0.1 mL PSG. Blood from a group of mice may be pooled.
4. An equal volume of PSG 6:4 with 14% glycerol is added slowly to the mouse blood, mixed, and allowed to equilibrate for 5–10 min.
5. The mixture is dispensed in 0.25–0.4 mL aliquots into sterile 1 mL cryotubes which are placed in a polystyrene box (to slow the freezing process), frozen overnight at –70°C then transferred to liquid nitrogen.
6. Infective feeds can be stored frozen indefinitely in liquid nitrogen or for several weeks at –70°C.

12.3.3 Preparation of procyclic form trypanosomes

1. Procyclic trypanosomes are obtained by infecting tsetse with bloodstream form trypanosomes and dissecting midguts from the flies about 10 days post infection, taking care to keep the dissection procedure as sterile as possible.
2. Trypanosome infected midguts are simply placed in Cunningham's medium supplemented with 20% fetal calf serum at 25°C; gentamicin is added (120 mg/mL) to reduce the risk of bacterial infection.
3. Once established a procyclic culture can be maintained almost indefinitely by simply changing the supernatant medium (with medium containing only 20 mg/mL gentamicin) every other day.

12.3.4 Preparation of an infective feed for tsetse

(a) Infecting with bloodstream form trypanosomes

Infective feeds are prepared from frozen stabilates of trypanosomes suspended in defibrinated pig blood (Welburn and Maudlin, 1987). Infective doses may vary from 1.5×10^5 to 2.5×10^6 trypanosomes per mL.

1. Cryotubes are defrosted in a bottle containing PBS (phosphate-buffered saline) at RT – this is to avoid accidents associated with tubes bursting during the defrosting procedure.
2. One mL PSG is added slowly to the vial and then the contents are made up to 4 mL in a sterile centrifuge tube with PSG.
3. The tube is centrifuged at 1500 g for 10 min at 4°C, and the supernatant is removed from the tube leaving 0.5 mL remaining over the pellet.

4. The trypanosomes are resuspended, their viability is checked, and they are then added to fresh blood or serum with red cells.
5. The final concentration of trypanosomes in this infective feed is counted by phase-contrast microscopy (Herbert and Lumsden, 1976) and may be adjusted to give the required infective dose.

(b) Infecting with procyclic form trypanosomes

1. Infective feeds are prepared by adding 1 mL procyclic culture supernatant (containing about 10^7 trypanosomes/mL) to 4 mL defibrinated pig blood.
2. Trypanosomes may be concentrated prior to feeding by centrifugation of the culture supernatant at 2500 rpm for 10 min at RT.

12.3.5 Experimental infection of flies

(a) Handling newly emerged flies

1. Emerging flies should be caged and immobilized (by chilling briefly in the ice compartment of a refrigerator) then placed in a Petri dish on ice.
2. The wings should then be removed from each fly with a pair of scissors; this is an important safety step which eliminates the problem of escapees infecting laboratory workers. It is also essential that this step is taken with any control flies involved, even if they are not to be infected.
3. Flies should then be re-caged and returned to the insectary for 24 h before infection. Maximum infectivity will be achieved if the flies are infected at their first feed, in the so-called teneral state.

(b) Infection of flies through an artificial membrane

1. Teneral flies are placed in circular cages 12.5 cm diameter with netted sides.
2. Five mL infected blood (infective feeds are prepared as described above) is poured on to a sterile feeding plate and covered with a silicone membrane 15 cm diameter, and distributed around the membrane with a small roller.
3. The plate/membrane is then placed on a heating plate at 37°C and allowed to warm for a few minutes.
4. The cage containing the flies is then placed on the membrane and flies are allowed to feed for about 30 min. Feeding will be encouraged if the lights in the room are dimmed.
5. The cage is then removed from the membrane and, if infection rates are being measured, any flies not taking a feed are removed from the cage using a pair of fine forceps. Chilling of flies immediately after feeding should not be attempted as this will kill the flies.
6. Cages of infected flies are then returned to the insectary.
7. The plate/membrane is soaked in water to kill the remaining trypanosomes, washed in clean water and sterilized for further use by placing in an oven at 100°C overnight (Notes 1–4).

12.3.6 Dissection of infected tsetse

When looking for mature infections, salivary glands should be dissected out about 28 days post-infection.

1. Flies should be chilled as described above and placed on a microscope slide in a drop of medium or saline.
2. Using a dissection microscope and fine dissection forceps the abdomen is opened and the gut and salivary glands dissected out (Buxton,1955, for details). Organs are examined for trypanosomes by phase contrast microscopy (×400).
3. The transmissibility of a trypanosome stock is quantified as the Transmission Index (TI%): %TI = salivary gland infections/midgut infections ×100.

12.3.7 Routine feeding of flies and issues relating to biosafety

Following infection, flies should be offered clean blood at least four times per week. Flies may be routinely maintained on defibrinated blood fed through an artificial membrane. *When working with human infective pathogens disposable gloves should be worn throughout to avoid accidental infection. Great care should be exercised when handling cages containing infected flies.* Treatment for accidental infections of *T. b. rhodesiense* is a dangerous and sometimes fatal procedure.

NOTES

1. *In vitro* feeding of tsetse: The *in vitro* feeding system developed for maintaining colonies of tsetse in the absence of live hosts offers great advantages for the production and maintenance of infected flies. This removes the problem of having an infected host ready for each infective feed and also ensures that the flies in replicated experiments are given the same infective dose of trypanosomes. The maintenance of flies after infection presents problems when animals are used; re-use of the same host animal/s risks re-infection of flies. Additional techniques for *in vitro* feeding of tsetse are described in detail in Chapter 5.
2. Artificially increasing infection rates in tsetse: For experimental purposes it is often desirable to obtain as many infected flies as possible. This can be achieved relatively simply by interfering with the fly's lectin-based defence system: (i) Inhibitory carbohydrates are added to the infective feed or for the period of establishment of the procyclic infection (1–5 days). D+ glucosamine and GlcNac increase infection rates of bloodstream form and procyclic forms in *G. m. morsitans* while addition of D+ galactose with D+ glucosamine increases infection rates in *G. p. palpalis*. Carbohydrates are prepared in saline to give a final concentration of 0.015 to 0.03 M in defibrinated pig blood. A maximum of 200 μL carbohydrate to 5 mL of blood is generally used. Where saline is used as a control solution, 200 μL saline is added to 5 mL blood. (ii) Infection rates can be increased with procyclic forms simply by removing serum from the infective feed. Infective feeds will simply comprise saline, red cells (washed 5 times in saline) and procyclic trypanosomes. Flies should be starved for 1–2 days post-infection before being fed on whole blood.

3. Salivary gland infections: To obtain the maximum numbers of salivary gland infections using *T. b. rhodesiense* it is advisable to use only male tsetse which normally produce significantly more infections than females (Dale *et al.*, 1995).
4. Infection of older flies: Once tsetse have been fed for the first time after emergence it becomes increasingly difficult to infect them but these flies may be infected if carbohydrates are added to the nonteneral feeds as described above for teneral flies.

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CHAPTER THIRTEEN

Infection of triatomines with Trypanosoma cruzi

Eloi S. Garcia and Patricia de Azambuja

13.1 INTRODUCTION

The scientist who discovered American trypanosomiasis (Chagas' disease), Carlos Chagas, not only wisely recognized the disease as a clinical entity, but also identified its causative agent (*Trypanosoma cruzi*) and its haematophagous (triatomine) insect vector (Chagas, 1909). Despite the fact that the reduviid vectors of the parasite have been well identified for more than eight decades, the understanding of the invertebrate cycle of the parasite remains remarkably limited. In *T. cruzi,* for instance, the trypomastigote stage does not multiply. It is the result of transformation of the parasite population that has been dividing as epimastigotes or amastigotes. Furthermore, the infective forms for both vertebrate and invertebrate hosts are metacyclic and bloodstream trypomastigotes, respectively.

Chagas' disease, a major endemic disease in Latin America, is dependent on a high degree of interaction between triatomine vectors and mammalian hosts. The parasite's life cycle displays quite distinct morphological and functional forms, alternating between dividing stages (epimastigotes in the insect vector and amastigotes in mammalian cells) and nonreplicative but infective forms (metacyclic trypomastigotes in the insect vector and bloodstream trypomastigotes in mammals). During the invertebrate phase, *T. cruzi* undergoes differentiation twice in the insect gut vector. The first is the transformation of trypomastigotes ingested with the blood meal into epimastigotes, which proliferate in the gut; the second is the differentiation of epimastigotes into metacyclic trypomastigotes, which are eventually eliminated together with faeces and urine and are able to infect vertebrate hosts (for reviews, Brener, 1973; Zeledon, 1983; Garcia and Azambuja, 1991). Drastic biological alterations accompany the transformation of the epimastigote into the trypomastigote of the invertebrate host cycle, including the capacity of the metacyclic trypomastigote to survive in mammals, in contrast to the epimastigotes which are destroyed by the complement system (Nogueira *et al.*, 1972).

The degree of natural infectivity of a vector species to a strain of *T. cruzi* depends on the susceptibility of the triatomine species to become infected when feeding on

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an infective blood meal. Another important aspect which merits mention with regard to the susceptibility of triatomines to *T. cruzi*, is the adaptation of a strain of parasite to develop more readily in an insect vector species. The virulence of *T. cruzi* strains to mammals and invertebrate hosts can change during long-term *in vitro* maintenance of the parasite (Bice and Zeledon, 1970; Chiari, 1974). The inability of some strains of *T. cruzi* to develop in certain species of triatomines may depend on intrinsic qualities of either parasite or insect. There seems to be a tendency of local vectors to be more susceptible to strains of *T. cruzi* from the same geographical areas. Thus, if a parasite strain does not grow well in one triatomine species, it is possible that it may develop in different species or in the same species from a different geographical region (Garcia *et al.*, 1986).

13.1.1 Culturing of *Trypanosoma cruzi*

T. cruzi can be maintained in the laboratory in many different ways, including both *in vivo* and *in vitro* systems. Thus, it may be kept in experimental animal hosts by routine serial syringe blood passages and in a number of different triatomine species. It can also be maintained in culture, either in cell-free media or in cell lines, such as mice or human monocytes and macrophages.

A variety of culture media have been employed for growing different forms of *T. cruzi*. In our laboratory the maintenance of *T. cruzi* for infection of the insect vector incorporates the following techniques: (i) epimastigotes are produced by routine serial passage of the parasite to axenic culture media, usually Liver Infusion Tryptose (LIT) medium or Brain Heart Infusion (BHI) medium; (ii) metacyclic trypomastigotes are cultured in old LIT or BHI medium or, more preferably, in Triatomine Artificial Urine medium – TAU medium (Contreras *et al.*, 1985); (iii) other trypomastigotes are obtained from mammalian tissue cultures infected from LIT and/or BHI culture-derived parasites; and (iv) bloodstream trypomastigotes are taken from infected mice.

The choice of technique for culture and production of *T. cruzi* will depend on the number of insects to be infected and the specific objectives of the investigation. It should again be emphasized that if *T. cruzi* strains are extensively passed *in vitro*, they can loose their infectivity to both vertebrate and invertebrate hosts. Thus, routine serial passage of *T. cruzi* in animals (mice, rats, hamsters or guinea pigs) and insect vectors will assure that *T. cruzi* strains retain their infectivity and virulence.

13.1.2 Rearing of infected triatomines

Triatomines are obligatory haematophagous insects throughout their entire life; thus infection depends on the insect vectors feeding on blood containing *T. cruzi*. A more conventional technique for infecting triatomines involves allowing them to feed either directly on infected living animal hosts (such as mice, rats and guinea pigs) with a high parasitaemia or on an infected blood meal through a membrane device (Chapter 6). Precautionary measures must be in place in order to insure that infected individuals do not escape.

13.2 MATERIALS

13.2.1 LIT medium

1. 4.0 g NaCl2; 0.4 g KCl; 8.0 g Na_2HPO_4; 2.0 g Glucose; 5.0 g Tryptose (Difco or Oxoid); 5.0 g Liver infusion broth (Difco); 25 mg Hemin (Sigma), dissolved in 1.0% NaOH; 100 mL Serum (ox, calf or fetal bovine serum).
2. Concentrated HCl.
3. EKS-Seitz membranes.
4. Millipore membranes and filters.
5. Ampicillin or penicillin and streptomycin.
6. Double-distilled H_2O (ddH_2O).
7. Erlenmeyer flasks of 200–250 mL capacity.

13.2.2 BHI Medium

1. 1 g Folic acid (Sigma) dissolved in 1.0% NaOH; 100 mg Hemin (Sigma) dissolved in 1.0% NaOH; 37 g Brain Heart Infusion (BHI, Dfco); 100 mL Serum (ox, calf or fetal bovine serum).
2. Millipore membranes and filters.
3. Ampicillin or penicillin and streptomycin.
4. ddH_2O.
5. Erlenmeyer flasks of 200–250 mL capacity.

13.2.3 TAU medium

1. 190 mM NaCl; 17 mM KCl; 8 mM Na_2HPO_4; 2 mM $MgCl_2$; 2 mM $CaCl_2$.
2. New-born calf serum.
3. Millipore membranes and filters.
4. Milk dilution flasks (surface area 25 cm^2).
5. Assay tubes of 16 × 15 mm (Kimax).

13.2.4 Tissue culture

1. 0.13 M NaCl.
2. Mammalian cell lines (epithelial kidney cell lines (VERO), LLC/-MK2 and Bovine Embryo Skin and Muscle (BESM) or any sensitive type of culture cells).
3. DME medium (sterilized by filtration in Millipore membrane).
4. Fetal calf (FCS) or calf serum (CS) (purchased sterile).
5. Plastic cell culture flasks (sterile).
6. Plastic Petri dishes (sterile).

13.2.5 Living hosts and feeding membrane device

The techniques for the use of living vertebrate hosts (mice, rats, hamsters and guinea pigs) and/or membrane device for feeding and maintaining large-scale colonies of triatomines in the laboratory are described in detail in Chapter 6.

13.3 METHODS

13.3.1 Production of epimastigotes

(a) Preparation of LIT medium

1. Dissolve the media components (Section 13.2.1, step 1) and serum in ddH_2O, adjust the pH of the medium to 7.2 with concentrated HCl and complete the volume to 1 L with ddH_2O.
2. Filter either through EKS-Seitz membranes, under negative pressure, or through a Millipore filter, under positive nitrogen pressure (before filtering, the medium has to be heated for 60 min at 68°C for serum inactivation; if the serum is inactivated only at 56°C, this can cause parasite clumping).
3. If necessary use either ampicillin (10 mg/mL) or penicillin plus streptomycin (100 000 U and 100 000 μg/L, respectively).
4. Filtered and inactivated LIT medium can be stored for several months at –20°C.

(b) Preparation of BHI medium

1. Dissolve the BHI in ddH_2O water and autoclave the solution.
2. In parallel, 100 μL of hemin and 100 μL folic acid solutions are added to 1 mL of ddH_2O and filtered through a Millipore filter.
3. The sterile serum is heated for 60 min at 68°C for serum inactivation.
4. To the sterile BHI medium add both the sterile hemin and folic acid mixture and inactivated serum. Sterile ddH_2O water is added to make up to 1 L.
5. Again, if necessary use either ampicillin (10 mg/mL) or penicillin plus streptomycin (100 000 U and 100 000 μg/L, respectively).
6. Sterile and inactivated BHI medium can be stored for several months at –20°C.

(c) Culture T. cruzi *in LIT or BHI media*

1. Parasites should be cultured at 26–28°C using Erlenmeyer flasks of 200–250 mL capacity containing about 50 mL of medium.
2. Ensure that the parasite culture has adequate aeration. If necessary, the culture can be gently shaken.
3. Epimastigotes in the log phase of growth or early plateau cultures can be used to seed new cultures. The average generation time is variable and depends on the parasite strain. Usually, the time taken for the number of parasites to double is about 30–40 h.
4. Cultures can reach up to 1×10^7 cells/mL within 7–10 days of incubation.
5. Cell number is scored by counting the parasites in a Neubauer chamber or an electronic cell counter after appropriate dilution.

13.3.2. Production of metacyclic trypomastigotes *in vitro*

(a) Preparation of TAU medium

1. Dissolve all salts in ddH_2O and adjust the pH of the medium to 6.0.
2. Filter the medium through Millipore membranes.

(b) Cultivation of parasites in TAU medium

1. Parasites from 6–10 days' old cultures in LIT medium are centrifuged at 10 000 g for 10 min at 10°C and cultured in freshly prepared LIT medium for 48 h at 27°C.
2. Cells are harvested as described above and incubated for 2 h at 27°C with TAU.
3. Parasites (95–100% epimastigotes) are diluted to a final concentration of 10^6 cells/mL with TAU medium supplemented with 10% new-born calf serum (TAUS medium), and incubated for different times at 27°C in 10 mL aliquots in milk dilution flasks without shaking. The *in vitro* metacyclogenesis is monitored by counting cells in a Neubauer chamber and depends on the strain of *T. cruzi* utilized.
4. Usually during the first 2 days of incubation cell aggregation can occur. After 6–8 days of incubation, titres as high as 80–90% of metacyclics are obtained.

13.3.3 Production of trypomastigotes in mammalian tissue culture

All mammalian cell types experimented with to date are sensitive to invasion and infection by the trypomastigote form of *T. cruzi* (Andrews and Colli, 1982). Therefore, the lack of host cell specificity facilitates the production of trypomastigotes in tissue culture since several easily maintained permanent cell lines serve as an appropriate host-cell. Usually, epithelial kidney cell lines (VERO) (Earley *et al.*, 1967), LLC/-MK2 (Hull *et al.*, 1956) as well as Bovine Embryo Skin and Muscle (BESM) cell cultures, are used for the production of trypomastigotes.

1. Bloodstream trypomastigotes are maintained by routine serial syringe blood passages in mice inoculated intraperitoneally with 10^5–10^6 trypomastigotes/mouse.
2. Parasitaemia is followed during the first 3–4 weeks of infection by microscopic examination of fresh blood obtained from the tail of mice selected randomly from each group.
3. Blood from infected mice is aseptically harvested in the presence of 0.13 M NaCl and incubated for 60 min at 37°C.
4. Supernatant plasma, containing blood form trypomastigotes, is seeded into flasks (plastic cell culture flasks for large production of trypomastigotes or plastic Petri dishes for small numbers of parasites) containing monolayers of mammalian cells in DME-10% calf serum or fetal calf serum medium.
5. After 72 h at 37°C, the medium is aspirated, the monolayer is washed with PBS, and fresh DME is added with only 2% calf serum.
6. Trypomastigotes that have been released from infected cells normally appear after 2–3 days incubation at 37°C.
7. To obtain trypomastigotes free of cell debris and transitional forms, the culture medium is centrifuged and incubated at 37°C for 60 min. After this period, pure trypomastigotes are recovered in the supernatant.

13.3.4 Production of bloodstream trypomastigotes

1. Infection with *T. cruzi* in mice, rats, guinea pigs or hamsters is maintained by serial intraperitoneal inoculation of parasites. Passages are repeated periodically,

depending on the time required by each particular strain to reach the parasitaemic peak.

2. Parasitaemia is evaluated by microscopic examination of fresh blood (Section 13.3.3) and the parasites counted in a Neubauer chamber.

13.3.5 Methods for infecting and quantifying *T. cruzi* infections in triatomines

In our laboratory we have frequently infected triatomines with a blood meal containing *T. cruzi* given through a feeding membrane device (Chapter 6). The parasite forms and the type of meal depend on the experimental objective. Metacyclic trypomastigotes, bloodstream trypomastigotes and trypomastigotes from tissue cultures, all of which are resistant to complement lysis, can be added directly to the blood meal. For infecting large numbers of triatomines, LIT and/or BHI media containing the parasites can be used as the meal or the insect vectors can be fed directly on the infected living hosts (Chapter 6). However, infecting triatomines through a membrane feeding apparatus is probably a simpler, safer, and more feasible method than using infected living hosts.

(a) Experimental infection of triatomines

1. In the case of using epimastigote forms for triatomine infections, the insect vectors must be fed on decomplemented blood [56°C for 60 min, otherwise the epimastigotes will be lysed by complement (Nogueira *et al.*, 1972)] containing parasites or even on the LIT and/or BHI media themselves.
2. Blood trypomastigotes, trypomastigotes from tissue culture or metacyclic trypomastigotes (obtained as previously described), are diluted with citrated or heparinized blood. Metacyclics to be used to infect triatomines must be washed twice in PBS and added in the meal to feed the insects.
3. Insects are allowed to feed on the infected meal for 30 min through the small artificial feeding apparatus previously described (Chapter 6).
4. The number of ingested parasites can be estimated from the calculated weight of ingested blood and by counting in a Neubauer chamber.

(b) Quantitation of parasites in infected bugs

For quantification of the infection within the insect, the entire gut of each insect is removed. To begin the dissection, an insect, or more if many guts are required, is pinned down in an appropriately sized dissecting dish 20 mm deep filled with wax or paraffin to a depth of 15 mm. Such a dissecting dish lasts almost indefinitely, the surface being restored by flaming with a Bunsen burner when covered by too many pinholes. These methods have been used in several quantitative papers from our laboratory (for reviews, Garcia *et al.*, 1989a,b; Gonzalez and Garcia, 1992).

1. Fix the insect with entomological pins inserted in positions which do not interfere with the subsequent dissection. For dissection, a dissecting microscope (magnification about 10×) is used.
2. With a pair of fine forceps (watchmaker's are suitable) the dorsal part of the abdominal wall is peeled back, without pulling it off, and pinned down to the

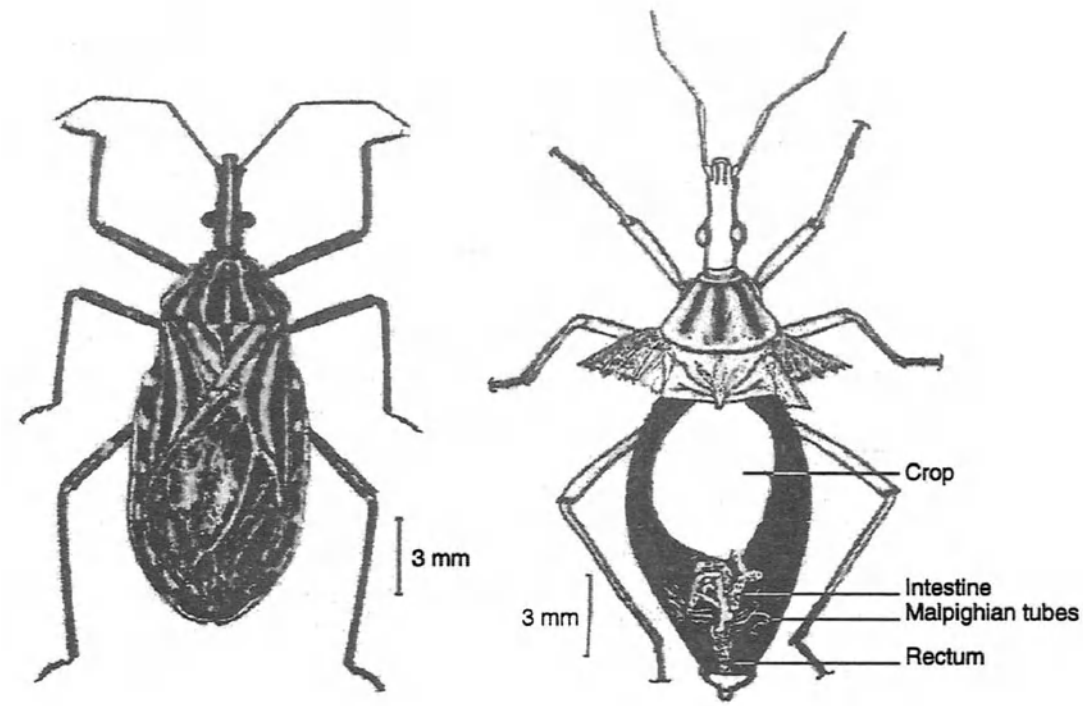


Figure 13.1 Adults of *Rhodnius prolixus*. Left, normal insect; right, insect with the dorsal part of the abdominal wall peeled. The gut (crop, intestine, rectum) and Malpighian tubules are clearly visible.

wax or paraffin. The fat body layer just inside the wall is conveniently pulled back and fixed at the same time.

3. Grab the anterior sac-like part (crop) – oesophagus end – of the gut and the end of the rectum with forceps. Gently remove together the crop, intestine (coiled part of the gut), and rectum, avoiding excessive tension on the gut. This particular stage of the dissection is critical and, unfortunately, is difficult to describe. The object is to take the whole gut so that it does not break apart. With some practice, on uninfected bugs, it is possible to become proficient in a short period of time.
4. Homogenize the whole gut in 1 mL of PBS using a small glass homogenizer. For safety reasons, this step is best performed in a biosafety cabinet to avoid exposure to potentially infective aerosols.
5. For evaluation of the number and morphological forms of the parasites use a Neubauer chamber.

13.3.6 Method for producing metacyclic trypomastigotes in triatomines

To produce large-scale metacyclic trypomastigotes of *T. cruzi* in the insect vector it is important to choose a strain of parasite which grows well in the particular triatomine vector selected for the experiment.

1. Infected fifth-instar larvae (fed in the third- and fourth-instar with infected blood) are also allowed to gorge until replete with uninfected blood (the infected feedings can be through a membrane feeding device or directly with living animal hosts).
2. For collection of metacyclic trypomastigotes in the clear urine or in the dark faeces, each insect, just after feeding, is placed head side up, in an 1.5 mL microfuge plastic tube which has been punctured at the bottom (Figure 13.2). This tube is set up inside another tube and kept overnight at 27–28°C. The excreted materials (pure urine or the mixture of urine and faeces) can be collected at different time intervals in the second tube after centrifuging the whole system if necessary.
3. Under these conditions, depending on the *T. cruzi* strains, the excreta can be quantitatively collected without loss and it is possible to obtain up to 1.5×10^7 metacyclic trypomastigotes/mL of excreta using about 70–90 infected insects.

13.3.7 Methods pertaining to biosafety concerns

In our laboratory the following rules are strictly adhered to in all procedures involving *T. cruzi*, whether in infected mammals, in infected vectors, or in *in vitro* culture systems.

1. All personnel handling either infected insects or *T. cruzi* cultures are trained in general laboratory procedures to work with pathogens and taught techniques especially required for manipulation of the parasite.
2. All personnel receive background knowledge about the parasites and close supervision when they first start to handle them.
3. During days of experiments with infected insects, access to the laboratory is restricted only to those personnel working on the parasites.

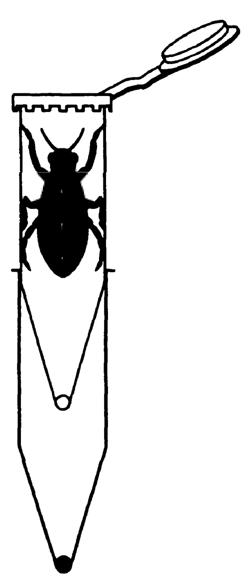


Figure 13.2 *Rhodnius* 'toilet'. Experimental arrangement used to collect pure urine and excreta (urine plus faeces) of *Rhodnius prolixus*.

4. Infected vertebrate and invertebrate hosts must be handled with gloves and work with these animals should be carried out, preferably in an isolated room on a surface covered with absorbent paper.
5. To prevent any accident or escape, the infected insects are placed inside plastic containers that are arranged inside larger plastic jars.
6. Dead infected insects or animals are very carefully and safely disposed of (usually insects are kept in iodine/ethanol solution before autoclaving and vertebrate hosts are placed in bags before autoclaving and incineration).
7. Personnel handling *T. cruzi* must use gloves, shoes and, if necessary, a face mask. Pipetting by mouth is strictly forbidden.
8. All nondisposable laboratory glassware and other materials that have came into contact with infected insects and vertebrate hosts are decontaminated with iodine-based detergents and germicidal solutions before washing and sterilizing.
9. Inform the local medical authorities in case of any accident with *T. cruzi.*

NOTES

1. The nomenclature of the distinct morphological forms of parasites (epimastigote and trypomastigote) is based on cell shape and motility – trypomastigotes are easily recognized by (i) the undulating membrane which makes the parasite appear to have a tender and typical body motility; (ii) the shape of the kinetoplast; (iii) spatial reorganization of the complex kinetoplast-basal bodies-flagellar reservoir – the point of emergence of the flagellum from the cell body and whether the kinetoplast is anterior (epimastigote) or posterior (trypomastigote) to the nucleus; and (iv) elongation or reduction of the free flagellum (Brener, 1973; 1979).

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Part Three
Basic Methods in Isolating, Cloning and Characterizing Nucleic Acids and their Products

CHAPTER FOURTEEN

Isolation of genomic DNA

Christos Louis

14.1 INTRODUCTION

It has been claimed that there exist at least as many methods of isolating nucleic acids as there are molecular biology laboratories (Jowett, 1986). This is surely an understatement, since my experience tells me that every graduate student will tend to introduce a new, usually irrelevant modification that, he or she hopes, will bring him to the top of the list of the most cited scientific authors. What is common to all of these methods is the care that has to be taken to obtain large molecular weight DNA, (i) by avoiding the action of contaminating DNases and (ii) by applying the least possible mechanical forces in order to minimize the shearing of the extremely long nucleic acid.

This chapter presents two methods of genomic DNA isolation that are in use by many people in my laboratory. Also, a method to isolate mitochondrial DNA from a relatively small number of flies will be presented. References for some other methods can be looked up in practically any publication describing experiments using genomic DNA of *D. melanogaster* or in laboratory manuals such as Sambrook *et al.* (1989), Jowett (1986) and Ashburner (1989). Furthermore, Chapter 16 describes a purification procedure for the preparation of DNA to be used in Cot curve analysis and Chapter 21 describes a simplified protocol for the rapid isolation of DNA that can be used in PCR amplification. Both procedures described here for genomic DNA isolation derive (with minor modifications) from groups working with *Drosophila melanogaster* which, for the purpose of this chapter, should be considered a *bona fide* insect!

The first method deals with the 'routine' extraction of DNA, while the second one can be applied if one wishes to obtain especially high molecular weight material for the construction of genomic libraries. The first protocol can be applied to as little starting material as one single fly or mosquito. One should bear in mind, when numbers are given in 'flies', that for all practical purposes *Sarcophaga* is indeed much larger than *Drosophila*! Also, the homogenization of larger insects is much more difficult than the homogenization of mosquitoes, their chitin cover being much thicker. The ratio of tissue to homogenization buffer will also affect the purity of the isolated DNA. When the ratio is high, the DNA will be less pure and it will often not be digestible by restriction enzymes. Moreover, eye pigments tend to copurify with the nucleic acids, but fortunately they do not usually affect the quality of the DNA. Contaminating RNA can be a nuisance both because it will mask some bands following electrophoresis, and also because it will affect spectrophotometric

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measurements of DNA concentration (as will the eye pigments). RNA can be removed by RNase treatment either during the isolation of the DNA, or during (and even after) the digestion with restriction enzymes. As briefly mentioned, one has to constantly think of the fact that the use of excessive shearing forces will result in low molecular weight DNA, which sometimes may be good for the purpose of Southern analysis, but which will not be of good quality if one wants to use the genomic DNA for the construction of a library. This makes it necessary to use cut-off pipette tips or Pasteur pipettes whose tips have been broken to obtain a wider bore (Note 2); it is also mandatory to avoid vortexing and hard shaking during the phenol extraction steps.

Finally, a word on the preparation of mitochondrial DNA. Since this is a small, circular and supercoiled DNA molecule, it is much more stable and not too prone to be degraded by mechanical stress. The novice should, thus, be warned not to follow the sometimes vigorous example of colleagues working with this kind of DNA, especially at the stage of phenol extraction.

14.2 MATERIALS

1. Homogenization buffer A: 10 mM Tris-HCl, pH 8.0, 60 mM NaCl, 10 mM EDTA, 0.15 mM spermine, 0.15 mM spermidine, 5% sucrose. Store at –20°C.
2. Extraction buffer A: 1.25% SDS, 300 mM Tris-HCl, pH 8.0, 0.1 M EDTA, 5% sucrose. Store at RT.
3. Homogenization buffer B: 100 mM Tris-HCl, pH 8.0, 50 mM NaCl, 50 mM EDTA, 1% SDS, 0.15 mM spermine, 0.15 mM spermidine.
4. Homogenization buffer C: 30 mM Tris-HCl, pH 7.5, 10 mM EDTA, 0.25 M sucrose.
5. MT buffer: 10 mM Tris-HCl, pH 8.0, 10 mM EDTA, 150 mM NaCl.
6. Phenol: Re-distil phenol at 160°C. Equilibrate the phenol by repeated extractions with 50 mM Tris, pH 8.0, until the pH is above 7.6. Add 8-hydroxyquinoline as an antioxidant and store at 4°C.
7. Chloroform/Isoamyl alcohol: Mix them in a ratio of 24:1. Store at RT.
8. Proteinase K: Add H_2O to the newly opened vial at a final concentration of 20 mg/mL. Store at –20°C.
9. Sodium acetate: 2.5 M solution. Adjust to pH 5.2 with acetic acid. Autoclave. Store at RT.
10. TE: 10 mM Tris-HCl, pH 7.4, 10 mM EDTA. Store at RT.
11. RNase: Heat RNase solution (1 mg/mL in 200 mM sodium acetate pH 5.0) for 15 min in a boiling water bath to destroy contaminating DNases. Store at –20°C.
12. Ethanol (for precipitation): Store at –20°C.
13. SDS (sodium dodecyl sulfate): 20% working solution. Wear a face mask while weighing the SDS. Store at RT.
14. Potassium acetate: For 1 L mix 600 mL 5M Potassium acetate, 115 mL glacial acetic acid and 285 mL H_2O. Store at RT.
15. 500 mM EDTA stock solution. Bring the pH to neutral while dissolving, otherwise the EDTA will not go into solution. Adjust to pH 7.0 with NaOH. Store at RT.

14.3 METHODS

14.3.1 Simple DNA extraction

Adult *Drosophila* should be used as a measure for the volumes to be used. Homogenization of a single to a few flies should be performed in 50–100 μL of buffer, while for 50–100 insects the volume should be increased to about 2 mL. Larger volumes have to be used accordingly for larger amounts of starting material (larger insects or a large number of small ones). Homogenization of less than 10 flies is best achieved in a plastic microfuge tube using either a heat-sealed Pasteur pipette or a yellow micropipette tip as a pestle. For larger numbers of flies, a glass homogenizer with a loose-fitting Teflon pestle should be employed. Under no circumstances should an electric motor be used at high speeds to drive the pestle, although its use at low revolution number may be of help for the homogenization of the insects. Several workers prefer to first grind the insects with pestle and mortar in liquid nitrogen before (or even instead) of the homogenization, and this may be especially helpful for large insects. Should several different sources of DNA be processed simultaneously, the homogenizer and pestle should be rinsed well with water between each of the preparations to avoid cross-contamination.

1. Gently homogenize the insects (or the powdered insects if they were first ground to powder in liquid nitrogen) in Homogenization buffer A.
2. Transfer to a plastic centrifuge tube and add an equal volume of Extraction buffer A. Use a microfuge tube for one to a few insects and larger tubes with a screw cap for larger volumes. Add proteinase K to a final concentration of 200 μg/mL. Mix gently and incubate at 37°C for 60 min.
3. Extract with phenol, followed by phenol-chloroform/isoamylalcohol (1:1) and chloroform/isoamylalcohol. *Wear gloves during the extraction steps!* The extractions should be very gentle, slowly inverting the sealed tube by hand for about 5–10 min and mixing the two phases. Alternatively mix the contents by using a rotator at low speed. Separate the phases by centrifugation at low speed. One can either discard the (lower) organic phase and use the same tube for every subsequent extraction step (cheaper), or sequentially transfer the aqueous phase into a new tube with a wide bore pipette (cleaner).
4. Add 1/10 vol of 2.5 M sodium acetate. Add two volumes of ice-cold (–20°C) ethanol and mix. Leave for 30–60 min at –20°C. Spin down the DNA by centrifugation (10 min at 8000 g). Discard the supernatant.
5. Add 100 μL of 70% ethanol to the pellet, gently swirl the tube and repellet the DNA by centrifugation. Discard the supernatant and invert the tube. Leave on a paper towel for a few minutes and dry briefly in a desiccator.
6. Redissolve the pellet in TE. If the DNA is difficult to dissolve, break the pellet loose from the bottom of the tube and heat to 37°C. Use about 10 μL of TE for a single fly, and 0.5 mL for 100 flies or the equivalent in other insects. Check the quality of the DNA by electrophoresis.
7. If RNase treatment is required at this point, add RNase to 100 μg/mL and incubate at 37°C for 30 min. Phenol extract once as described in step 3 and repeat steps 4–6. Store at 4°C.

14.3.2 Extraction of high molecular weight DNA

1. Grind 0.5–1.0 g of insects in liquid nitrogen using a mortar and pestle. Transfer the powdered material into a 50 mL sterile plastic centrifuge tube with a screw cap.
2. Add 10 mL of Homogenization buffer B and proteinase K to a final concentration of 200 μg/mL. Incubate at 45°C for 1 h.
3. Phenol-extract with an equal volume of redistilled phenol. Invert the tube *gently* for 5–10 min and separate the phases by centrifugation at low speed.
4. Transfer the aqueous phase into a new tube with a wide bore Pasteur pipette (broken tip) and repeat the phenol extraction once.
5. Extract once with chloroform/isoamyl alcohol following the same precautions as in steps 3 and 4.
6. Add 1/10 vol. 2.5 M sodium acetate. Slowly add 2 vol of ice-cold (–20°C) ethanol by letting it drip along the wall of the tube. The DNA should start precipitating at the interphase.
7. Using, as a rod, a Pasteur pipette whose tip has been heat-sealed and turned to form a hook, start 'fishing' the precipitated DNA. Use either an electric motor to rotate the rod at very low speed (<200 rpm) or . . . your hand. You can get increasingly more DNA to precipitate by gently swirling the tube, obtaining progressive mixing of the two phases.
8. When most DNA is around the rod, transfer it into a new sterile tube and leave the rod standing until most ethanol has dripped and the DNA is 'dry'. Transfer again into a new tube and dissolve in 10 mL of TE.
9. RNase-treat the DNA for 1 h at 37°C with pancreatic RNase at a concentration of 100 μg/mL.
10. Repeat steps 3–8. Estimate the concentration and the quality of the DNA by electrophoresis. The concentration (but not the quality) can be measured more accurately by spectroscopy at A_{260}. Store the DNA at 4°C.

14.3.3 Mitochondrial DNA extraction

This method (Tamura and Aotsoka, 1988) is basically an adaptation of the alkaline lysis procedure routinely used for the isolation of plasmid DNA. Its main advantage is the fact that it avoids the lengthy (and expensive) use of CsCl gradients such as, for example, the method described by Lansman *et al.* (1981).

1. Homogenize up to 100 *Drosophila* equivalents in 1 mL of homogenization buffer C using an electric motor at high speed. Transfer the homogenate into a microfuge tube and spin at low speed (800×g) at 4°C to pellet the nuclei and debris.
2. Spin the supernatant at high speed (12000×g) to pellet the mitochondria.
3. Resuspend the crude mitochondria in 50 μL of MT buffer. Add 100 μL of freshly prepared 0.18 N NaOH containing 1% SDS. Vortex the tube and store on ice for 5 min.
4. Add 75 μL of potassium acetate solution at 4°C. Vortex and leave on ice for an additional 5 min.
5. Spin in a microfuge at high speed for 5 min in the cold. Phenol-extract the supernatant with an equal volume of phenol-chloroform/isoamyl alcohol (1:1). Vortex

extensively. Separate the phases by centrifugation and precipitate the mtDNA by adding 2 vol of ice-cold ethanol. Wash the pellet with 70% ethanol and dry in a desiccator. Dissolve in 50–100 μL of TE. RNase treat before restriction digestion. Store at –20°C.

NOTES

1. Although less prone to nuclease degradation than RNA, avoid as much as possible any possible contamination with DNases. Use new plastic ware and preferably bake the glassware for 4 h at 250°C.
2. To make a wide bore Pasteur pipette hold the narrow end about 2–3 cm from the tip *inside a thick layer of paper tissues* and break it off. Use only the pipettes with a 'roundish' hole. The cutting can be helped by first scratching with a diamond glass cutter. For small volumes of DNA, one can alternatively cut off the end of a blue micropipette tip using a new razor blade.
3. Capped polypropylene tubes should be used for the phenol extraction steps. If microfuge tubes are to be used, they should be checked first that they don't leak organic solvents, since this is a fact in several cheaper brands.
4. Phenol should be equilibrated at pH 8.0, since DNA will tend to go to the interphase (or even the organic phase) at lower pH. Avoid taking the lipid layer at the interphase or any of the lower phase. If this happens, repeat the step.
5. Rather than 'fishing out' the high molecular weight DNA, some people prefer to dialyse extensively for up to 48 h against TE. In this case, one should bear in mind that the DNA solution will be viscous (depending on the concentration and quality), and that it will increase in volume, given the low ionic strength of the TE. One should compensate for this by using a dialysis tube cut larger than necessary.
6. 'Good' DNA should be visible on an ethidium bromide stained agarose gel (0.6%) as a single band migrating with an apparent size of >20–25 kb. Should a smear be visible, the DNA can be transferred to the waste basket and steps 1–9 have to be repeated! Use standard dilutions of unrestricted bacteriophage λ for the determination of genomic DNA concentrations.

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CHAPTER FIFTEEN

Isolation and analysis of RNA from insect vectors

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15.1 INTRODUCTION

RNA exists in three 'classical' forms in all cells: rRNA, tRNA and mRNA. The latter carries a copy of the information from a protein-coding region of the DNA to the ribosome where it directs the order of amino acids to be incorporated into a specific polypeptide. The examination of mRNA transcripts in insect vectors is therefore of fundamental importance in the study of gene expression and many key investigations depend on the isolation and purification of undegraded RNA. These include cDNA cloning (Chapter 19), the study of the relative abundance of transcripts by Northern blotting (Chapter 20), RT-PCR (Chapter 21), differential display (Chapter 22) and the localization of transcripts in tissues by *in situ* hybridization (Chapter 23).

Since the population of transcripts vary in different cell types as well as in the stage of development, one needs to establish which tissue is to be examined and at what time or whether a complete range of developmental stages is necessary to study the expression of a specific gene.

RNA is chemically and biologically more labile than DNA, particularly at temperatures greater than 65°C and in the presence of alkali. It is also susceptible to degradation by the resilient RNases that exist not only in the cells being processed but also in the laboratory environment. Stringent measures are therefore needed if one is to isolate RNA molecules intact.

This chapter will outline the precautions needed when handling RNA, the isolation and purification of total RNA from insect tissues, poly(A)$^+$ RNA purification and analysis of RNA by dot–blot hybridization.

15.2 MATERIALS

15.2.1 Preparation of reagents and equipment

1. Diethyl pyrocarbonate (DEPC): Make fresh as a 0.1% v/v solution in water. *DEPC is a potential carcinogen and should be handled with care!*
2. H_2O_2.

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15.2.2 Isolation of total RNA by guanidium thiocyanate/CsCl

1. GT I buffer: 4 M guanidium thiocyanate, 1% β-mercaptoethanol (0.14 M), 0.1 M Tris-Cl pH 7.5. To make: Dissolve 50 g guanidium thiocyanate in 10 mM Tris-Cl pH 7.5. Adjust volume to 100 mL and filter through Whatman No 1 filter. Add 1 mL β-mercaptoethanol before use.
2. Sarcosyl.
3. Caesium chloride/EDTA: Mix 96 g CsCl in 90 mL 0.01 M EDTA pH 7.5. Add DEPC to 0.1% and leave to stand. Autoclave and allow to cool before adjusting the volume to 100 mL with DEPC-treated water.
4. TE: 10 mM Tris-Cl pH 7.6, 1 mM EDTA.
5. Sodium dodecyl sulfate (SDS).
6. 3 M sodium acetate pH 5.2.
7. Absolute ethanol: Analar grade.

15.2.3 RNA isolation from small quantities of material

1. GT II buffer: 4 M guanidium thiocyanate, 25 mM sodium citrate, 0.5% sarcosyl, 100 mM β-mercaptoethanol. This can be prepared in advance as before without the β-mercaptoethanol.
2. 2 M sodium acetate pH 4.0.
3. Water saturated phenol (nucleic acid grade).
4. Chloroform:isoamyl alcohol (49:1 v/v).
5. Isopropanol.

15.2.4 Poly(A)$^+$ purification

1. Oligo(dT) cellulose (Boehringer Mannheim).
2. Oligo(dT) loading buffer: 0.5 M NaCl, 1 mM EDTA, 0.1% SDS, 10 mM Tris-Cl pH 7.5.
3. Elution buffer: 1 mM EDTA, 0.05% SDS, 10 mM Tris-Cl pH 7.5.
4. 0.1 N NaOH, 5 mM EDTA.

15.2.5 Dot–blot hybridization

1. 20×SSC (per L): 175.3 g NaCl, 88.2 g sodium citrate.
2. 100× Denhardt's solution (per L): 20 g ficoll, 20 g polyvinylpyrrolidone, 20 g bovine serum albumen (fraction V).
3. Formaldehyde (supplied at 37% or 12.3 M). *This is a potential liver carcinogen and should be used in a fumehood!* Check that the pH is neutral before use; otherwise deionize over a monobed resin.
4. Deionized formamide.
5. 20×SSPE: 3.6 M NaCl, 200 mM NaH_2PO_4 pH 7.4, 20 mM EDTA pH 7.4.
6. Hybridization solution: 50% deionized formamide, 5×SSPE, 2× Denhardt's solution, 0.1% SDS, 100 mg/mL denatured and sonicated nonhomologous DNA, e.g. salmon sperm DNA.

7. ^{32}P Labelled nucleotides (Amersham).
8. Manifold e.g. BRL Hybri-Dot™.
9. Whatman 3mM filter paper.
10. Nitrocellulose (or nylon) filter.
11. Vacuum source.

15.3 METHODS

15.3.1 Preparation of reagents and equipment for RNA work

In order to prepare a good sample of RNA, it is crucial that all contaminating RNase activity is removed. In addition, intrinsic levels of RNase activity must be curtailed upon disruption of the cell since this results in the liberation of these sequestered enzymes from damaged organelles. Failure to scrupulously observe the precautions listed below will undoubtedly yield a useless, degraded RNA sample.

1. Wear gloves at all times, even during the preparation of reagents and equipment. The oils in the fingertips of the investigator are a potentially rich source of RNase! Gloves should also be changed frequently as they come into contact with contaminated surfaces.
2. General plasticware, polypropylene products (pipette tips and microfuge tubes), glassware, mortar and pestle, etc. should be soaked in a solution of DEPC (0.1% in water) for at least 12 h before autoclaving for 15 min at 15 psi. Glassware can also be baked at 180°C for 3–4 h to remove RNase.
3. Serologically sterile plasticware can be used without further treatment.
4. All stock solutions (with the exceptions in Note 1) can be treated by the addition of DEPC to 0.1% and left for 12 h before autoclaving as above. It may be useful to keep solutions in the warm autoclave for a few hours after completion of the cycle to remove all traces of DEPC (Note 2).
5. Electrophoresis tanks should be cleaned with detergent, rinsed with water, dried with ethanol and filled with 3% H_2O_2. After 10 min, rinse thoroughly with DEPC-treated water.
6. Keep reagents and equipment for RNA work separate for that use. Aliquot stock reagents into working volumes to minimize contamination and discard aliquot after use.

15.3.2 Isolation of total RNA by guanidium thiocyanate/CsCl

Many protocols for the isolation of total RNA often include the use of guanidium thiocyanate since it is a strong chaotropic reagent causing deproteinization and membrane disruption in addition to being an effective inhibitor of endogenous RNAse. It is particularly useful when dealing with tissues having a high RNase content.

If one has a large amount of starting material, particularly from the larger insect vectors, the best approach is to use guanidium thiocyanate followed by purification of the resulting RNA by caesium chloride centrifugation (Chirgwin *et al.*, 1979; Glisin *et al.*, 1974; Ullrich *et al.*, 1977). RNA has a greater buoyant density (>1.8 g/mL) than

the usual contaminating macromolecules (DNA and protein) and will form a glassy pellet at the bottom of the tube.

However, in many instances, particularly with smaller insects such as sandflies or specific tissues, there may be insufficient starting material to even contemplate CsCl gradients and a simpler method is described in the next section. This approach is also useful for processing a large number of samples simultaneously.

For both methods, whole organisms or dissected tissues can be frozen in liquid nitrogen and stored at –70°C prior to the extraction. Several authors claim that this freezing step also improves the yield because it leads to the inactivation of cellular nucleases.

The isolation of RNA using guanidium thiocyanate and its purification on a CsCl density gradient is described below.

1. Place tissue in a sterile mortar and freeze by adding liquid N_2. Grind to a powder using a sterile pestle. Add more liquid N_2 if necessary.
2. Pulverized tissue is placed in a homogenizer and 5 vol of GT I buffer is added. Homogenize thoroughly (Note 3).
3. Add sarcosyl to a final concentration of 0.5%. Centrifuge at 5000 g for 10 min at RT to remove insoluble debris.
4. Transfer supernatant to a clean tube. If the sample has a high level of mucopolysaccharide or glycosamino glycans, it may be necessary to add CsCl to the sample at this stage (Note 4).
5. Draw sample into a hypodermic syringe fitted with a 23 G needle. Layer sample onto a cushion of 5.7 M CsCl, 0.01 M EDTA (pH 7.5) in a clear ultracentrifuge tube. Mark the top of the cushion on the outside of the tube. Table 15.1 lists some commonly used ultracentrifugation rotors and the conditions used. For different rotors refer to Note 5. Centrifuge at 20°C for the specified time. Do not allow the centrifuge chamber to cool as the CsCl will precipitate.
6. At the end of the run, turn off the centrifuge brake before decelerating the rotor to prevent disturbing the gradient.
7. Use a sterile pipette to remove liquid above the level of the cushion (containing cellular DNA and protein). Use a fresh pipette to remove liquid to within 1/2 cm from the bottom of the tube taking care not to disturb the pellet.
8. With a sterile razor blade, cut off the top of the tube just above the level of the remaining liquid that can be removed with an automatic pipette. Carefully invert the tube on a pad of tissues and allow to drain (Note 6).
9. Reinvert tube. Check that the translucent pellet has not fallen out. Fill with 70% EtOH. Invert and drain away as before.
10. Air-dry the pellet. Dissolve in an appropriate volume of TE pH 7.6 containing 0.1% SDS (Table 15.2) by repeatedly drawing into an automatic pipette. If the pellet is difficult to resuspend, freezing and thawing or homogenization may help (Note 7).
11. Transfer the solution to a microfuge tube. Rinse the ultracentrifuge tube with 25 μL TE and add to the sample.
12. Precipitate the RNA by adding 0.1 M sodium acetate pH 5.2 and 2.2 vol of absolute ethanol and leave at –20°C overnight.
13. Recover by centrifugation at 12 000 g for 10 min at 4°C. Wash in 70% ethanol and air dry.
14. Resuspend pellet in a small volume of DEPC-treated H_2O. Measure the absorbence at A_{260} and A_{280} to determine the quantity and quality of the prepa-

Table 15.1 Centrifugation details for isolating RNA from insects

Rotor	*CsCl pad (ml)*	*Homogenate (ml)*	*Rotor speed* (rpm × 1000)*	*Time of run*† *(h)*
SW50.1	1.3	3.6	47	8
			42	12
SW60	1.1	3.2	41	10
SW30.1	2.0	5.8	29	15
SW41	3.3	9.7	30	23
SW28	9.5	28.5	27	26

* Maximum rotor speed for tubes 1/4 full of 5.7M CsCl.
† Time required for pelleting based upon the clearing factor of each rotor at a given speed.

Table 15.2 Volumes to be used for redissolving RNA prepared from insects

Volume of homogenate	*Volume of TE/SDS*
3.3 ml	100 µl
10 ml	300 µl
26 ml	1 ml

ration. (1 A_{260} unit = 40 µg RNA, A_{260}:A_{280} should be between 1.9 and 2.2 if the sample is free of protein.)

15. RNA can be stored in small aliquots at –70°C in DEPC-treated water but avoid repeated freeze–thaw cycles. For long-term storage, it is best stored as an ethanol precipitate. To convenient sized samples, add 3 vol of ethanol and store at –70°C. When required, add 0.1 vol of 3 M NaOAc pH 5.2 and recover by centrifugation as before.

15.3.3 RNA isolation from small quantities of material

The following method (Chomczynsk and Sacchi, 1987) is suitable for RNA extraction from small amounts of starting material or simultaneous isolation from several samples. As little as 3 mg of tissue can be used in this protocol which gives good quality RNA.

1. Grind tissues in liquid nitrogen as in Section 3.2.
2. Place powder in an homogenizer and add 1 mL GT II buffer.
3. Transfer suspension to a 15 mL polypropylene tube and add (in order): 0.1 mL 2 M sodium acetate pH 4.0, 1 mL H_2O-saturated phenol, 0.2 mL chloroform:isoamyl alcohol. Cap the tube and mix thoroughly after each separate addition. Shake vigorously for 10 s after all the reagents have been added (Note 8). Cool on ice for 15 min.

4. Spin at 10 000 g for 20 min at 4°C. Transfer the aqueous phase to a clean tube.
5. Add an equal volume of isopropanol and store at –20°C for at least an hour.
6. Centrifuge at 10 000 g for 20 min at 4°C. Carefully discard the supernatant.
7. Dissolve the RNA pellet in 300 μL GT II buffer and transfer to a microfuge tube.
8. Reprecipitate the RNA with an equal volume of isopropanol and leave to precipitate and recover as before.
9. Wash the pellet twice in 500 μL 75% ethanol and air dry.
10. Redissolve the pellet in a small volume of DEPC-treated water or 1 mM EDTA, pH 8 and determine the yield by the absorbence at A_{260}.
11. Store in convenient sized aliquots at –70°C either resuspended or, better still, as an ethanol precipitate.

15.3.4 Isolation of poly(A)$^+$ RNA

In a sample of total RNA, the fraction composed of mRNA is relatively small (~16%). It may be desirable, if one has sufficient material, to enrich the sample in mRNA for cDNA synthesis or to increase the sensitivity of Northern blotting, S1 nuclease and RNAse protection assays. Most eukaryotic mRNAs have a 3′ poly(A) terminal that is added to the transcript post-transcriptionally (Nevins and Darnell, 1978). This is thought to facilitate transport of the mature mRNA out of the nucleus and to help stabilize it in the cytoplasm. However, it should be noted that not all mRNAs are polyadenylated (for example, histone mRNA).

The poly(A) tail lends itself for the separation of mRNA from other RNA species by affinity methods. The traditional method was to use an oligo(dT) cellulose column (Aviv and Leder, 1972) but, in recent years, a number of quicker variations have been developed. These include: (i) direct mixing of oligo(dT) cellulose and total RNA; (ii) paramagnetic beads with oligo(dT) attached; (iii) messenger affinity paper; and (iv) biotinylated oligo(dT) and precipitation of poly(A) complexes with avidin or streptavidin.

A brief outline of poly(A)$^+$ RNA purification by oligo(dT) column chromatography is given below.

1. Resuspend oligo(dT) in oligo(dT) loading buffer (high salt). Use 0.6 g oligo(dT)/mg RNA.
2. Plug a sterile syringe or pipette tip (depending on the amount of oligo(dT)) with glass wool and apply the slurry. Wash separately with (i) 3 vol of H_2O; (ii) 3 vol of 0.1 N NaOH, 5 mM EDTA; (iii) H_2O until pH of effluent is ~7; (iv) Re-equilibrate with oligo(dT) loading buffer.
3. Heat RNA to 65°C for 5 min. Add an equal volume of 2× oligo(dT) loading buffer and load onto the column at RT.
4. Wash column with at least 5 vol of 1× oligo(dT) loading buffer until the A_{260} of effluent is ~0.
5. Apply elution buffer and monitor A_{260} readings of the eluted fractions.
6. Precipitate with 0.1 vol 3 M sodium acetate, pH 5.2 and 2.5 vol of ethanol and store at –20°C overnight. Recover by centrifugation.
7. Regenerate oligo(dT) by repeating from steps 2(ii) to 4 without loading an RNA sample.

15.3.5 Dot–blot hybridization

The electrophoretic separation of RNA, the transfer of the material to filters (Northern analysis) and the hybridization protocols are described in Chapter 20. Here, I describe dot–blots and slot–blots, methods that allow rapid detection and quantitation of a specific target RNA sequence in purified RNA samples and cell lysates (Kafatos *et al.*, 1979; White and Bancroft, 1982). This is achieved by reading densitometric tracings of the resulting autoradiograph.

Suitable positive and negative controls must be included to demonstrate hybridization specificity since background levels of nonspecific binding may be a problem with this technique.

1. Wet the nitrocellulose in water and soak in 20×SSC for 1 h.
2. Clean the manifold in 0.1 N NaOH and rinse well in sterile water.
3. Place two sheets of Whatman 3MM filter paper (soaked in 20×SSC) onto the manifold. Place the nitrocellulose on top and smooth out any air bubbles. Assemble the manifold and connect to a vacuum unit.
4. Fill the holes with 10×SSC and apply a gentle suction. When all the fluid has passed through, turn off the vacuum, refill the holes as before and reapply the suction.
5. Prepare RNA samples (in 10 μL H_2O) by adding 200 μL formamide (100%), 7 μL formaldehyde (37%), 2 μL 20×SSC. Heat at 68°C for 15 min and chill on ice. Add 2 vol of 20×SSC. Up to 20 μg RNA may be used.
6. Load samples onto manifold and apply gentle suction. When all the samples have passed through, rinse with 1 mL of 10×SSC.
7. Continue the vacuum for 5 min to dry the nitrocellulose.
8. Remove the nitrocellulose and air-dry. Bake for 2 h at 80°C.
9. Hybridize to radiolabelled probe as described for the hybridization of Northern blots in Chapter 20 but use long prehybridization times (6–24 h).

With probes having a specific activity $>5 \times 10^8$ cpm/μg, it is possible to detect mRNAs that are present at approximately 5 copies per cell if 20 μg of total cellular RNA is applied to a single slot (Sambrook *et al.*, 1989).

NOTES

1. Tris, SDS and β-mercaptoethanol should not be treated with DEPC. Instead, they can be made with autoclaved DEPC-treated water and autoclaved again.
2. DEPC is destroyed by autoclaving (to ethanol and CO_2). All traces of DEPC must be removed from the reagents or else it will cause carboxymethylation of RNA.
3. Homogenization during the RNA extraction shears the nuclear DNA and prevents the formation of an impenetrable mat on the top of the CsCl cushion which might block the sedimentation of RNA to the bottom of the tube. Homogenization increases the yield of RNA by a factor of ~2. For small amounts of tissue, homogenization can be ideally carried out using a microfuge tube and a glass pestle. If a homogenizer is not available, DNA can be sheared by drawing the preparation several times through a 23 G needle attached to a sterile hypodermic syringe.

4. Mucopolysaccharides and glycosamino glycans may form an occlusion within the gradient that can be prevented by adding 0.5–1 g CsCl/mL lysate. Centrifugation times must then be reduced to stay within safe limits.
5. For rotors that differ from those mentioned in the text, it is necessary to calculate the equivalent clearing time using the clearing factor of the rotor at the maximum speed allowed for a tube 1/4 filled with 5.7 M (1.7 g/mL) CsCl (Rotors and tubes for preparative ultracentrifuges: An operator's manual. Beckman Instruments, Palo Alto, California, 1982). Fixed angle rotors allow shorter centrifugation times but the RNA will be pelleted along the side of the tube and will come into contact with the upper protein containing portion of the gradient.
6. Removing most of the ultracentrifugation tube after the gradient separation prevents contamination of the RNA pellet with DNA or protein that can adhere to the walls of the tube.
7. If the RNA is difficult to dissolve, freeze and thaw the sample twice and heat to 45°C. This usually breaks up pellets and allows them to dissolve rapidly. Alternatively, pellets can be homogenized in TE/SDS solution. If glycogen is present, this can be removed by selectively precipitating the RNA at high salt concentration. Add 2 vol of 4.5 M sodium acetate pH 7.0, mix and leave at –20°C. Recover by centrifugation.
8. The use of phenol and chloroform under acidic conditions causes partitioning of the RNA into the aqueous phase and DNA and protein into the organic phase.

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CHAPTER SIXTEEN

Determination of insect genome size and complexity by reassociation kinetics

Melanie J. Palmer and William C. Black IV

16.1 INTRODUCTION

In 1968, Britten and Kohne introduced C_0t curve analysis to examine the reassociation kinetics of genomic DNA. Using this technique they were able to estimate genome size and, more importantly, demonstrate that eukaryotic genomes consist of a complex mixture of repetitive and unique DNA sequences. In addition to protein coding genes, genomes contain large blocks of noncoding sequences, some of which are repetitive. Repetitive DNA is categorized as either highly repetitive (~1000 – 100 000 copies) or moderately repetitive (2–~1000 copies) (reviewed in Hartl, 1991; Lewin, 1990).

Analysis of the reassociation kinetics of a genome simultaneously provides information on genome size and the relative frequencies of snap-back ('satellite'), highly repetitive, moderately repetitive and unique sequences. It can also provide information on the complexity and abundance of highly and moderately repetitive sequences. Unfortunately, the use of C_0t curve analysis has been limited, with the result that currently we have a poor understanding about genome size and organization in the largest and most species-rich phylum, Insecta. Table 16.1 lists the genome size and organization patterns analysed thus far in arthropods. The genome sizes vary widely, between approximately 5×10^7 base pairs (0.05 pg) to 5×10^9 base pairs (5 pg) (Lewin, 1980, 1990).

The advent of the polymerase chain reaction technique (PCR) has revolutionized biology and has allowed the isolation of DNA and RNA sequences from a variety of organisms without prior knowledge of the gene sequence. Unfortunately, utilization of most molecular biological techniques still requires a fundamental knowledge of genome size and organization. For example, when constructing genomic libraries, genome size is essential to calculate the number of recombinant clones that must be generated (Clarke and Carbon, 1976) to provide complete coverage of the genome. It is also essential to estimate copy number of repeat families. Although other techniques have been developed (Cockburn and Mitchell, 1989; Kumar and Rai, 1990; Vinogradov, 1994) to estimate both DNA amount and the frequency of repetitive sequences, C_0t analysis is still the only technique which can simultaneously estimate genome size, complexity, and abundance of repetitive DNA.

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Table 16.1 Genome size, organization and composition in those arthropods in which DNA reassociation kinetics have been examined

Species	*Genome size*		*Interspersion*	*Percent composition**				*Reference*
	pg	*bp*	*type*	*SB*	*HR*	*MD*	*UN*	
Drosophila melanogaster	0.18	1.7×10^8	Long	3	14	9	74	Crain *et al.*, 1976b
Apis cerana	0.19	1.8×10^8	Long	5	0	6	89	Jordan and Brosemer, 1974
Chironomus tentans	0.20	1.9×10^8	Long	0	0	10	90	Wells *et al.*, 1976
Tribolium castaneum	0.21	2.0×10^8	Long	20	3	15	62	Brown *et al.*, 1990
Apis mellifera	0.22	2.1×10^8	Long	7	0	0	92	Crain *et al.*, 1976a
Anopheles quadrimaculatus	0.24	2.3×10^8	Long	4	0	16	80	Black and Rai, 1988
Anopheles gambiae	0.27	2.6×10^8	Long	6	0	33	61	Besansky and Powell, 1992
Megachile rotundata	0.30	2.9×10^8	Long	4	0	7	89	Jordan and Brosemer, 1974
Schizaphis graminum	0.40	3.9×10^8	Long	2	0	20	78	Ma *et al.*, 1992
Bombyx mori	0.53	5.1×10^8	Short	10	14	21	55	Gage, 1974
Culex pipiens	0.54	5.2×10^8	Short	11	38	29	22	Black and Rai, 1988
Sarcophaga bullata	0.61	5.9×10^8	Long	4	9	6	81	Samols and Swift, 1979
Aedes aegypti	0.83	8.0×10^8	Short	8	12	20	60	Warren and Crampton, 1991
Aedes albopictus (Calcutta)	0.86	8.3×10^8	Short	10	17	37	36	Black and Rai, 1988
Musca domestica	0.89	8.6×10^8	Short	21	23	22	34	Crain *et al.*, 1976a
Antheraea pernyi	1.00	9.7×10^8	Short	5	30	30	35	Efstratiadis *et al.*, 1976
Amblyomma americanum	1.08	1.0×10^9	Long	4	18	42	36	Palmer *et al.*, 1994
Aedes albopictus (Mauritius)	1.32	1.3×10^9	Short	10	31	27	33	Black and Rai, 1988
Aedes triseriatus	1.52	1.5×10^9	Short	10	44	31	16	Black and Rai, 1988

* Percentages may not equal 100% due to rounding.
SB, snap-back sequences; HR, highly repetitive sequences; MD, moderately repetitive sequences; UN, unique sequences.

16.1.1 DNA reassociation kinetics

The reassociation of two complementary strands of DNA in a complex mixture of single strands depends upon random collision and follows second-order kinetics (Britten *et. al*, 1974). The rate of the reaction is described by the equation:

$$\frac{dC}{dt} = -kC^2 \tag{16.1}$$

Where C is the concentration of DNA that is single-stranded at time *t* and *k* is the reassociation rate constant. If the equation is integrated between the limits of the initial concentration of DNA, C_0 at time $t = 0$, and the concentration C_t remaining single-stranded after time *t* then,

$$\frac{Ct}{Co} = \frac{1}{1 + k\,C_0 t} \tag{16.2}$$

With equation 16.2, the reassociation of any DNA can be described by the rate constant *k*, given in units of L/mol s or in the form of its reciprocal, C_0t, given in mol s/L. The term C_0t describes the progress of the reassociation of a single DNA sequence in a fixed volume of solution during *t* seconds of incubation. Reassociation of DNA is usually plotted as the fraction of DNA that remains single-stranded (C_t/C_0) against the $\log_{10}$ of the C_0t. The form of this curve for *Escherichia coli* (*E. coli*) genomic DNA is shown in Figure 16.1a.

When the reaction is half-complete:

$$\frac{Ct}{Co} = \frac{1}{2} = \frac{1}{1 + k\,C_0 t_{1/2}} \tag{16.3}$$

$C_0t_{1/2}$ is equal to the reciprocal of *k* and is often reported in comparisons of reassociation rates among different DNA sequences.

Equations 16.1 and 16.2 describe the reassociation kinetics of a single DNA sequence over time. However, prokaryotic and eukaryotic genomes consist of complex mixtures of genes. Because prokaryotic genomes often contain little repetitive DNA, unique sequences often reassociate at a constant rate and reassociation kinetics generally follow equation 16.2. However, eukaryotic genomes often contain a significant amount of repetitive DNA. If we dissolve a eukaryotic genome in solution, repetitive sequences will, by definition, be at a higher concentration and, according to equations 16.1 or 16.2, will reassociate at a faster rate. It follows that highly repetitive sequences will reassociate at a greater rate than moderately repetitive sequences and these will reassociate at a greater rate than unique sequences. Therefore an equation that accurately describes the reassociation of eukaryotic DNA is a composite of three reassociation equations:

$$\frac{Ct}{Co} = \frac{Co(\text{high})}{1 + k(\text{high})Co(\text{high})t} + \frac{Co(\text{mod})}{1 + k(\text{mod})Co(\text{mod})t} + \frac{Co(\text{high})}{1 + k(\text{high})Co(\text{high})t} \tag{16.4}$$

Where *k*(high), *k*(mod) and *k*(unique) are the reassociation constants of highly repetitive, moderately repetitive and unique sequences respectively and C_0(high), C_0(mod) and C_0(unique) are the initial concentrations of highly repetitive, moderately repetitive and unique sequences. A plot of equation 16.4 is shown in Figure 16.1b. The composite curve at the top of Figure 16.1b is the usual form of reassociation seen in eukaryotic genomes. By fitting reassociation data from the composite

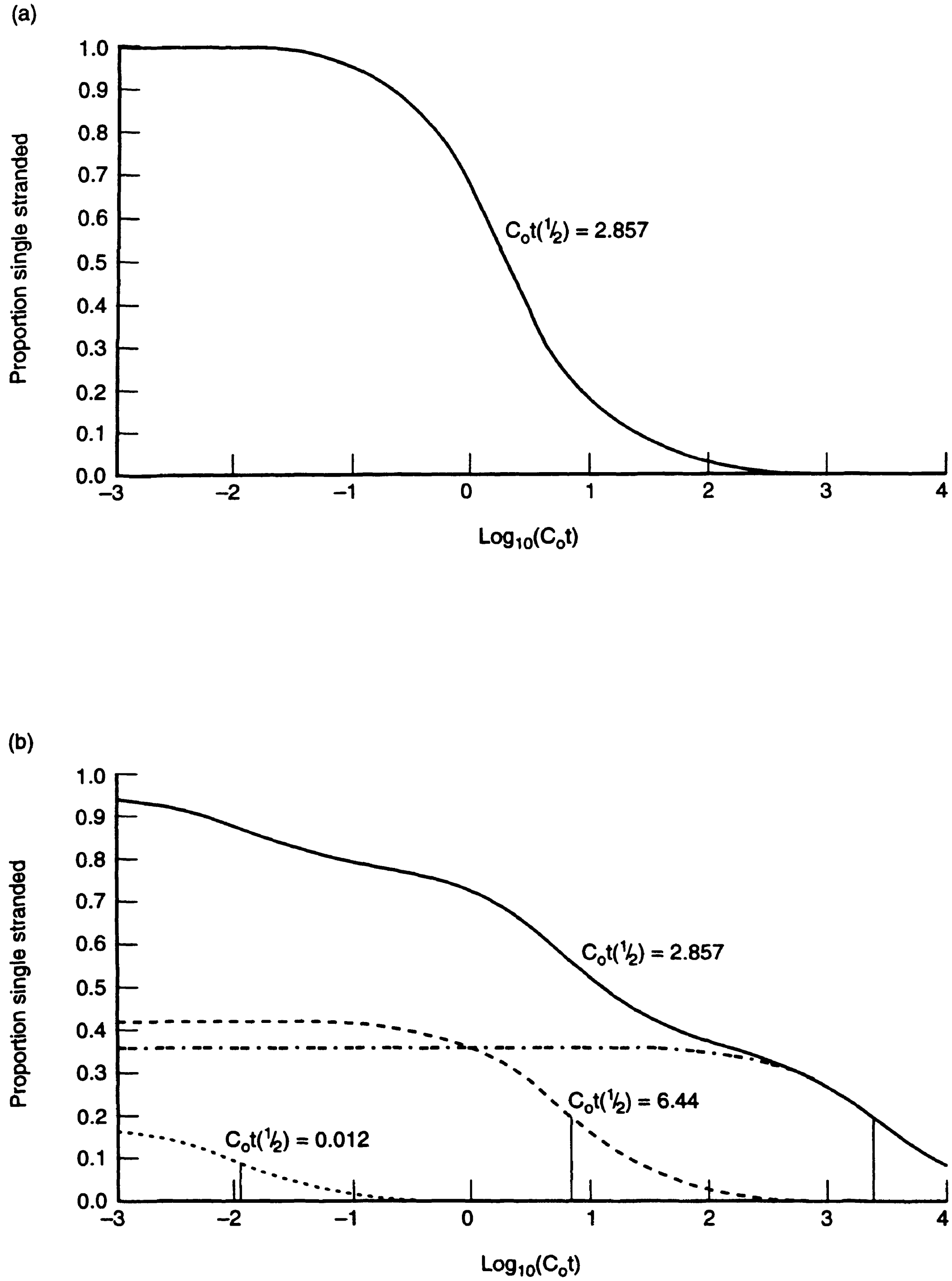


Figure 16.1 DNA reassociation curves. (A) Reassociation of *E. coli* DNA plotted as a function of the $\log_{10}$ C_0t values. (B) Reassociation of a eukaryotic genome plotted as a function of the $\log_{10}$ C_0t values. The solid line presents the composite curve. Dashed and dotted lines represent reassociation curves of the individual unique (-..-), moderately repetitive (– – –), and highly repetitive (...) components.

curve to the model in equation (16.4) using multiple regression analysis, all three reassociation rates and concentrations can be estimated. At the base of Figure 16.1b are shown the reassociation curves and associated $C_0t_{1/2}$ estimates for the highly repetitive, moderately repetitive and unique components. The estimates of $C_0t_{1/2}$ are reciprocals of *k*. Note that the y-intercept of each curve estimates the proportion of each type of DNA. Note also that these proportions do not add to 100% nor does the composite curve intercept the y-axis at 1.0. This is due to the 'snap-back' sequences described earlier. These are single strand pieces of DNA that form intrastrand pairing and reassociate at rates greater than interstrand pairing among highly repeated sequences.

The DNA in eukaryotic genomes typically reassociates from C_0t values as low as 0.0001–0.001 for highly repetitive or snapback sequences to 10 000 for single copy sequences. The $C_0t_{1/2}$ of the unique component of a genome increases with genome size. As the genome becomes more complex, the concentration of a given DNA sequence within a given mass of DNA is reduced and thus, the time required for reassociation is increased. If two species have equal concentrations of unique sequences, the species with the greater amount of repetitive DNA will have a greater $C_0t_{1/2}$. The $C_0t_{1/2}$ of a genome is therefore proportional to its complexity. Thus, the complexity of any DNA can be determined by comparing the $C_0t_{1/2}$ of its unique component with that of a standard DNA of known complexity, such as *E. coli* (Lewin, 1990). Once the *k* of the unique component has been estimated as indicated above, the $C_0t_{1/2}$ is computed as the reciprocal of *k* and genome size is estimated using the proportion:

$$\frac{\text{Genome size}}{C_0t_{1/2}\ \text{unique DNA}} = \frac{4.2 \times 10^6\ \text{bp}\ (0.0044\ \text{pg})}{C_0t_{1/2}\ \textit{E. coli}} \tag{16.5}$$

Where $C_0t_{1/2}$ *E. coli* is estimated under the same conditions as those in which $C_0t_{1/2}$ was estimated for the arthropod species under investigation. One pg is equivalent to 0.965×10^9 bp (base pair).

From this information we can estimate the average frequency (f) of repeats in the highly repetitive and moderately repetitive components using the equation:

$$f = \frac{C_0t_{1/2}\ \text{of the unique fraction}}{C_0t_{1/2}\ \text{of the repetitive fraction}} \tag{16.6}$$

This indicates the relative complexity of the repetitive components. The $C_0t_{1/2}$ of a reaction indicates the total length of different sequences present and describes their sequence complexity or total length (in nucleotide pairs). If the highly repetitive DNA consists of a single sequence, then it will reassociate faster than a mixture of repetitive sequences. For example, Black and Rai (1988) observed that many copies of a highly repetitive element had accumulated in a Calcutta strain of *Aedes albopictus*.

Although repetitive sequences vary in amount and organization throughout all eukaryotes, two distinct organizational patterns are recognized (Davidson *et al.*, 1975). The most common type is short period interspersion in which single copy sequences (1–2 kb in length) are linked to short (0.2–0.6 kb) and to moderately long (1–4 kb) repetitive sequences. Long period interspersion consists of a pattern of long repeats (5–6 kb) that are interspersed with very long stretches of single copy sequences (>10 kb). Therefore, in patterns of long period interspersion only a very small fraction of single copy DNA is linked to repetitive sequences on 1–2 kb fragments.

When long and short fragments consist of similar sequences, as occurs in patterns of long period interspersion, the renaturation rate of long fragments can be predicted from the following equation (Wetmur and Davidson, 1968):

$$\frac{\text{Rate of reassociation}}{\text{of long fragments}} = \frac{\text{Rate of reassociation}}{\text{of short fragments}} \times \sqrt{L/S} \tag{16.7}$$

However, if the long DNA fragments contain unique and repetitive sequences (short period interspersion), the repetitive sequences will reassociate rapidly, and the reassociation rate of the long fragments will be faster than predicted. Therefore, by simply comparing *k* values obtained for long and short DNA fragments, the interspersion pattern of repetitive DNA can be determined.

16.1.2 General strategy

There are nine general steps involved in the construction of a C_0t curve. These are listed below to provide the reader with a general overview of the steps involved. Details of each step are then provided in the methods section.

1. Purchase several mg of *E. coli* DNA and calf thymus DNA to standardize procedures and to estimate genome size (equation (5) above). At the same time, isolate several mg of arthropod genomic DNA.
2. Shear *E. coli* and arthropod DNA to produce short DNA fragments. Reserve some arthropod DNA to be sheared or digested to produce long DNA fragments.
3. Estimate the average fragment length of short and long DNA fragments.
4. Make stocks of 0.03 M, 0.12 M and 0.40 M sodium phosphate buffers (PB) and prepare hydroxyapatite (HAP) columns.
5. Resuspend long and short DNA fragments of *E. coli* and arthropod DNA in 0.12 M PB at low (~100 μg/mL) and medium (~500 μg/mL) concentrations to estimate reassociation at low (0.01–1) and medium (0–100) C_0t values. Resuspend long and short DNA fragments of arthropod DNA in 0.4 M PB at high concentration (~1 mg/mL) to estimate reassociation at high C_0t values.
6. Plan time points along reassociation curve so that a uniform spread of C_0t values will be obtained.
7. Denature DNA with heat. Initiate reassociation at 60°C for low and medium C_0t values and at 66°C for high C_0t values and stop reassociation reactions at planned time points to estimate reassociation at specific C_0t values.
8. Separate single- and double-strand fragments by HAP chromatography and estimate single- and double-strand concentrations with UV spectrophotometry.
9. Enter the percent reassociation for each C_0t value into a multiple regression analysis to estimate *k*.

Details of the preceding procedures are discussed in the following sections. Using these procedures it is possible to measure reassociation kinetics quickly, usually only requiring one large scale preparation of arthropod DNA.

16.2 MATERIALS

16.2.1 Genomic DNA isolation

1. Liquid nitrogen.
2. 2× Nuclear Isolation Buffer (2× NIB): 20 mM Tris, 120 mM NaCl, and 20 mM EDTA, pH 7.4.
3. 100 mM Spermine.
4. 100 mM Spermidine.
5. Mortar and pestle.
6. 10% Sarcosyl (w/v).
7. Triton-X 100.
8. Caesium chloride (CsCl).
9. 10 mg/mL Ethidium bromide (EtBr).
10. Water-saturated 1-butanol.
11. TE: 10 mM Tris (pH 8.0), 1 mM EDTA (pH 8.0).
12. 3M sodium acetate (NaOAC), pH 5.2.
13. 100% ethanol (EtOH).
14. 70% EtOH.

16.2.2 Producing long and short DNA fragments for reassociation

1. 2× Sonication buffer: 40 mM Tris, pH 8.0, 2mM EDTA, pH 8.0, 5 mM β-mercaptoethanol.
2. *E. coli* DNA (Sigma D2001).
3. Calf thymus DNA (Sigma D3664).

16.2.3 Estimation of average fragment length

1. Molecular weight markers: 100 bp ladder (BRL) and λ DNA digested with *Hin*dIII.
2. Electrophoresis grade agarose.
3. 10× Tris borate buffer (TBE): 900 mM Tris Borate, 20 mM EDTA).

16.2.4 Preparation of phosphate buffer stocks (PB)

1. 1 kg $NaHPO_4$ (monosodium phosphate).
2. 1 kg Na_2HPO_4 (disodium phosphate · heptahydrate).

16.2.5 Preparing sonicated DNA samples for Co*t* analysis

1. 3 M NaOAC, pH 5.2.
2. 100% EtOH.
3. 70% EtOH.
4. 0.12 M PB.
5. 0.4 M PB.
6. Mineral oil.

16.2.6 Denaturation and reassociation of DNA samples

1. Dry ice-ethanol bath.
2. 60°C and 66°C water baths.

16.2.7 Hydroxyapatite chromatography

1. Whatman G/F circles.
2. 105 μm nylon mesh (Small Parts Inc.).
3. 10 mL disposable or glass syringes (Beckman).
4. 18 G needles.
5. Hydoxyapatite (biogel HTP, Biorad).
6. 3 M NaOAC, pH 5.2.
7. 100% EtOH.
8. 70% EtOH.
9. 0.03 M PB.
10. 0.12 M PB.
11. 0.4 M PB.

16.2.8 An alternative method for C_0t curve analysis when the amount of arthropod DNA is limiting

1. Nick translation kit (Gibco-BRL).
2. Deoxycytidine 5′ triphosphate tetrasodium salt [5,5′-^{3}H] 15–30 Ci/mM (ICN or Dupont NEN).
3. G-50 sephadex (Sigma).
4. STE: 100 mM NaCl, 10 mM Tris, pH 8.0, 1 mM EDTA.
5. 0.03 M PB.
6. 0.12 M PB.
7. 0.4 M PB.
8. 10 μL microcapillary pipettes and a microcapillary pipette filler.

16.3 METHODS

16.3.1 Genomic DNA isolation

1. Quick freeze insects/arthropods in liquid nitrogen and store at –70°C until use.
2. Prepare 600 mL of 1× NIB by combining 300 mL of 2× NIB with 3.0 mL Triton-X 100, 0.9 mL 100 mM spermadine, 0.9 mL 100 mM spermine and 295.2 mL of sterile dH_2O.
3. Grind 2 g of frozen insects/arthropods to a fine powder with a mortar and pestle, adding liquid N_2 as needed to keep the tissue frozen.
4. Transfer to a 50 mL disposable centrifuge tube and allow the liquid N_2 to evaporate.
5. Add 40 mL of 1× NIB and thaw until liquefied.
6. Transfer to a 55 mL glass homogenizer and grind until there are no clumps of cells remaining.

7. Return the sample to the 50 mL disposable centrifuge tube and spin for 10–15 s at the highest setting in a table top centrifuge to remove debris. The objective is to pellet cuticle and debris while leaving nuclei in suspension.
8. Transfer the supernatant to a new tube and repeat step 7.
9. Transfer the supernatant to 50 mL high speed polypropylene centrifuge tube and centrifuge for 10 min at 4°C, at approximately 7000 **g** to pellet nuclei.
10. Discard the supernatant and thoroughly resuspend the crude nuclear pellet in a 2–4 mL volume of NIB using a 1 mL micropipettor. The sample can be vortexed, if needed, to get the pellet into solution.
11. Bring the volume to 30 mL and centrifuge again for 10 min at 4°C, at approximately 7000 **g** to pellet nuclei.
12. Discard the supernatant and thoroughly resuspend the nuclear pellet in 2 mL NIB as outlined in step 9.
13. Bring the volume to 14 mL with 1× NIB and add 4 mL 10% Sarcosyl to lyse the nuclei. Gently mix with a glass rod and incubate on ice for 10 min. The lysate should become viscous as the nuclei lyse.
14. Transfer the lysate to a small beaker or a 50 mL disposable centrifuge tube, slowly add 21.26 g CsCl, and gently stir or rock to dissolve the CsCl. The solution can be gently heated to aid dissolution.
15. Bring the volume to 26.75 mL with NIB, add 0.27 mL of 10 mg/mL ethidium bromide (EtBr), and rock for 20 min to obtain a homogeneous solution. If there are particulates in the solution, filter the solution through cheesecloth to remove debris before transferring to centrifuge tubes. **Ethidium bromide is a powerful mutagen. Please see Sambrook *et al.* (1989) for descriptions of proper handling and disposal.**
16. Using a wide bore Pasteur pipette, carefully transfer the lysate to two 16 × 76 mm ultracentrifuge tubes (Beckman quickseal or equivalent). It is critical that the tubes be full and that they be carefully balanced before sealing. If there is not sufficient lysate to fill the tubes to the recommended level, use paraffin oil to balance the tubes.
17. Seal the tubes, and spin at 45–50 000rpm for 16–18 h at 20°C in a vertical or near vertical rotor to separate DNA, RNA and proteins. Following ultracentrifugation, the genomic DNA will appear as a discrete band that is fluorescent under ultraviolet light.
18. Insert a 21 G needle into the top of the centrifuge tube to allow air to enter. Next, insert an 18 G hypodermic needle attached to a 3 or 5 mL disposable syringe above the band so that the open bevelled end is just above the DNA band. Carefully withdraw the viscous DNA band into the syringe. Remove the 18 G needle from the syringe and transfer the DNA solution to a 15 mL tube.
19. Remove the EtBr by performing several extractions with an equal volume of water-saturated 1-butanol. The EtBr will partition in the top butanol phase. Perform extractions until no pink colour remains. For a detailed description of collection of DNA from CsCl gradients and subsequent extraction of EtBr, see Sambrook *et al.* (1989)
20. Remove the CsCl by performing dialysis in several exchanges of TE buffer.
21. Following dialysis, estimate the DNA yield by measuring the optical density of a small, undiluted aliquot at A_{260}. The optical density × 50 μg/mL × volume of the DNA solution (after dialysis) will provide an estimate of yield.

22. Precipitate the DNA with 1/10 vol 3 M NaOAC (pH 5.2) and 2.5 vol 100% EtOH at –20°C for several hours to overnight. Following precipitation, pellet the DNA at high speed 5–6000 g for 30 min at 4°C.
23. Wash the DNA pellet with several mL 70% EtOH and centrifuge for 5 min at 7000 g to remove residual salt. Let the pellet air-dry and resuspend very gently in TE at a concentration of ~1 mg/mL. DNA should be resuspended while the pellet is still moist or it will be difficult to resuspend. DNA can be heated gently and/or rocked to aid dissolution.
24. Measure the optical density of the DNA at A_{260} and A_{280}. The A_{260}/A_{280} ratio should be close to 1.8 (Notes) and the concentration close to 1 mg/mL.
25. If desired, the integrity of the genomic DNA can be checked by electrophoresing 100 ng of undigested DNA through a 0.3% agarose gel using ~200 ng of *Hin*dIII restricted DNA as a size standard (Section 16.3.3 and Notes). Genomic DNA should run as a high molecular weight band with streaking upward of the 24 kb marker band. Significant smearing downward is indicative of DNA degradation.

16.3.2 Producing short and long DNA fragments for reassociation

To estimate the interspersion patterns of high and moderately repetitive DNA sequences, genomic DNA must be fragmented into long and short fragments and a C_0t curve generated for each size class. Although shearing of DNA by sonication is rapid, it is rather imprecise and results can vary considerably with different sonicators. Therefore, optimum conditions for producing DNA fragments in the short size range and long size range must be determined empirically. We strongly recommend that you first optimize sonication parameters for your sonicator using commercially supplied calf thymus and *E. coli* DNAs before proceeding with arthropod DNA. The production of longer DNA fragments in the size range of 1–10 kb is especially difficult and imprecise with sonication, so it is critical that you establish reproducible conditions before attempting to produce long DNA fragments with arthropod DNA (Figure 16.2).

1. To produce DNA fragments of 300–500 bp, dilute 1.5–2 mg of calf thymus DNA into 2× sonication buffer for a final concentration of 500 μg/mL (4 mL in a 15 mL polypropylene tube). It is important that all DNA be in solution for it to shear uniformly.
2. Quickly cool the DNA solution to 0°C in an ethanol-dry ice bath, and sonicate on the maximum power setting for 10 s.
3. Repeat the cycles of chilling and sonication until an average fragment length of 300–500 bp is obtained (~10–20 times). Because it is difficult to shear DNA into the size range of 300–500 bp, excessive sonication is not likely to be a problem.
4. To monitor the change in fragment length, remove a 2–4 μL aliquot (1–2 μg DNA) following each round of sonication, and place in a microcentrifuge tube. Add gel dye and electrophorese through a 1% agarose gel (Section 16.3.3 to monitor the change in fragment size following sonication).
5. Repeat steps 1–4 with *E. coli* and arthropod DNA using optimized conditions.
6. To produce DNA fragments of 500–10 000 bp, dilute 1.5–2 mg of calf thymus DNA to 500 μg/mL in 2× sonication buffer.
7. Chill the DNA solution to 5°C and sonicate on minimum power in 5 s bursts, 5–20 times with intermittent chilling.

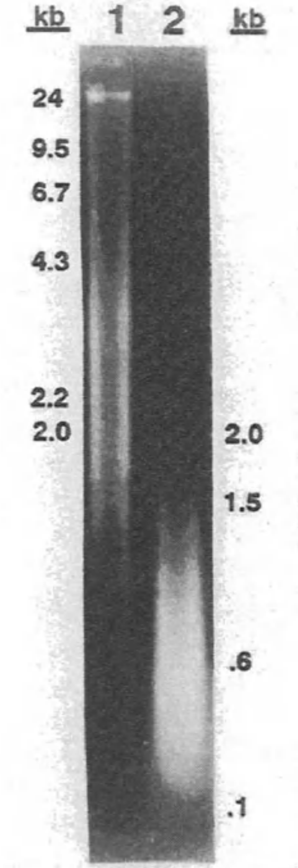


Figure 16.2 Agarose gel electrophoresis of sheared eukaryotic DNA. Lane 1: large DNA fragments with average fragment size between 2 and 4 kb. Please note that there is an additional population of large fragments ~24 kb that should be removed before renaturation; Lane 2: short DNA fragments with an average fragment size between 0.3 and 0.6 kb.

8. Following each round of sonication, remove a 2–4 μL aliquot (1–2 μg DNA) and place in individual microfuge tubes. Add gel dye and electrophorese through a 0.7% agarose gel along with a λ *Hin*dIII molecular weight marker to monitor the change in fragment size. Most DNA fragments should be in the range of 1–10 kb. If necessary, increase the power to shear DNA into this size range.
9. Repeat steps 6–8 with arthropod DNA using optimized conditions. It is not necessary to produce a long fragment population for *E. coli* DNA.

16.3.3 Estimation of average fragment length

The average fragment length will be used to determine the value of L/S in equation 16.7, which, in turn, will determine interspersion patterns of repetitive sequences. Since a distribution of fragments will be obtained, an exact value cannot be estimated, but this is not critical in using equation 16.7.

1. Electrophorese ~1 μg aliquots of short DNA fragments in a 1.2% agarose gel in 1× TBE (8.9 mM Tris borate, 0.2 mM EDTA) with a 100 bp molecular weight ladder (Gibco-BRL) to provide a standard for generating a molecular weight curve.

2. To estimate the size of the long DNA fragments electrophorese a 1 μg aliquot through a 0.7% agarose gel in 1× TBE with a molecular weight marker such as λ *Hin*dIII that will provide a range of both high and low molecular weights (20–0.5 kb).
3. Stain the gel with EtBr (0.5 μg/mL), place a ruler next to the molecular weight marker lane so that the 0 cm marker is aligned with the bottom of the well and photograph the gel. The distance each band in the molecular weight marker has migrated will be calculated by aligning the band with the ruler.
4. Generate a molecular weight curve by plotting the molecular weights of the molecular weight standard (X axis) against the distance migrated (Y axis) on semilog graph paper. To generate the curve, draw a line connecting all the points.
5. Draw lines to bracket the region that stains most intensely and then draw a line midway through the bracketed region and measure the distance migrated at that point.
6. Plot the distance migrated on the molecular weight curve and calculate the molecular weight by estimating where that point on the curve intersects the X axis.

16.3.4 Preparation of phosphate buffer stocks (PB)

DNA reassociations are carried out in sodium phosphate that is buffered to pH 7.0. The buffer increases the stability of the DNA during heating and cooling and the salt concentration determines the reassociation rate. Renaturation at low and intermediate C_0t values is routinely performed in 0.12 M PB (0.12 M with respect to phosphate and 0.18 M with respect to Na^+) at a temperature of 60°C. Renaturation at high C_0t values is performed in 0.4 M (0.6 M Na^+) at 66°C to drive the rate of renaturation.

1. Prepare 2 L of a 1 M solution of disodium phosphate (Na_2HPO_4) and 2 L of a 1 M solution of monosodium phosphate ($NaHPO_4{\cdot}7H_2O$).
2. To obtain a 1 M PB solution of pH 7.0, mix 1 M disodium (Na_2HPO_4) and 1 M monosodium phosphate ($NaHPO_4{\cdot}7H_2O$) in a ratio of approximately 57.7 to 42.3. If the pH is below 7.0 slowly add more 1 M Na_2HPO_4 until the pH reaches 7.0. If the pH is above 7.0 add more 1 M $NaHPO_4{\cdot}7H_2O$ until the pH drops to 7.0. You can also refer to Table B.11 (Sambrook *et al.*, 1989) for preparation of solutions of Na_2HPO_4 and $NaHPO_4{\cdot}7H_2O$ (pH 7.0).
3. Make appropriate dilutions of the 1 M PB stock in deionized water to obtain 0.03 M, 0.12 M, and 0.4 M phosphate buffers and pH to 7.0, if necessary. Two litres each of 0.3 M, 0.12 M, and 0.4 M phosphate buffers is sufficient to construct C_0t curves for *E. coli* as well as several arthropod species.

16.3.5 Preparing sonicated DNA samples for C_0t analysis

1. Following sonication and estimation of average fragment length, determine the yield of the remaining DNA by measuring the optical density of the solution at A_{260} and A_{320}. Correct for background by subtracting the absorbency at 320nm from that obtained at 260nm.
2. Each time point will require 30–40 μg of sonicated DNA. Low and medium C_0t values will require ten time points or approximately 400 μg DNA for each reas-

sociation. The high C_0t values will require 15 time points and approximately 600 µg DNA.

3. Precipitate 400–600 µg DNA for low, medium and high C_0t values with 1/10 vol 3 M NaOAC and 2.5 vol 100% EtOH at –20°C for several hours.
4. Recover the DNA by centrifuging at 6000 g for 20–30 min.
5. Wash the pellet in 70% EtOH and allow the pellet to air dry.
6. Resuspend the DNA in 0.12 M PB at approximate concentrations of 100 µg/mL and 500 µg/mL for low and mid C_0t values, respectively and in 0.4 M PB at 1–2 mg/mL for high C_0t values. Allow plenty of time for the DNA to go into solution!
7. Before aliquoting the DNA, verify the DNA concentration by diluting a small aliquot from low, medium, and high concentrations in water and measuring the A_{260} and A_{320}. Subtract the absorbency at A_{320} from A_{260} to correct for background. Make the same dilution of the appropriate PB buffer to use as a blank.
8. Dispense the DNA in 30–40 µg aliquots into separate microcentrifuge tubes (0.4–1.5 mL). Alternately DNA can be renatured in one large tube and aliquots removed at specific time points.
9. Layer each sample with several drops of mineral oil to prevent sample evaporation during renaturation.

16.3.6 Determining time points for reassociation

Because eukaryotic DNA usually renatures over a range of C_0t values spanning several orders of magnitude, it is necessary to perform renaturations at several different DNA concentrations and at different salt concentrations to obtain a complete renaturation profile between 10 and 90% double-stranded DNA. Reassociation of *E. coli* DNA will occur at C_0t values from 10^{-2} to 10^2. Reassociation of arthropod DNA will occur at C_0t values from 10^{-3} to 10^4.

1. *E. coli* DNA at low concentration (~ 100 µg/mL) will be renatured to C_0t values of 0, 0.01, 0.02, 0.05, 0.1, 0.5, 1.0, and 10.
2. *E. coli* DNA at medium concentration (~ 500 µg/mL) will be renatured to C_0t values of 0, 0.1, 0.5, 1.0, 1.5, 2.0, 4.0, 6.0, 8.0, 10, 20, 50, and 100.
3. Arthropod DNA at low concentration (~ 100 µg/mL) will be renatured to C_0t values of 0, 0.01, 0.02, 0.05, 0.1, 0.5, ,1.0, 1.5, 2.0, and 5.0.
4. Arthropod DNA at medium concentration (~ 500 µg/mL) will be renatured to C_0t values of 0, 0.1, 0.5, 1.0, 2.0, 5.0, 10, 50, 100, and 200.
5. Arthropod DNA at high concentration (~ 1–2 mg/mL) will be renatured to C_0t values of 0, 1.0, 5.0, 50, 100, 150, 200, 250, 500, 750, 1000, 2000, 4000, and 6000.
6. Use the optical density (A_{260} × dilution factor) obtained in section 3.5, step 6 in the following equation to calculate the time required to renature to a particular C_0t value in 0.12 M PB (low and medium C_0t curves): time (hours) = $C_0t \times 2/A_{260}$. For example, A_{260} (0.75) × dilution factor (1/10) = 7.5. Therefore, the time required to renature to Cot 200 is: time (hours) = 200 × 2/7.5 = 53.33 h or 53 h and 18 min. A word of caution: do not confuse absorbency values and DNA concentration. DNA concentration is determined by multiplying the *absorbence value* (A_{260}) by the dilution factor × 50 µg/mL.
7. Use the optical density (A_{260} × dilution factor) obtained in Section 16.3.5, step 6 in the following equation to calculate the time required to renature to a particu-

lar C_0t value in 0.4M PB (high C_0t curve): time (hours)/4.9409 = $C_0t \times 2/A_{260}$. A correction (4.9049) must be applied for the change in PB concentration to obtain an equivalent Cot value (EC_0t). Therefore, the time required to reach EC_0t 200 for DNA with an A_{260} of 7.5 would be 53.33/4.9049 = 200 × 2/7.5 = 10.8 h or 10 h 48 min.

16.3.7 Denaturation and reassociation of DNA samples

Because you will be collecting timepoints in rapid succession, we recommend that you initiate renaturation of only one C_0t curve at a time (i.e. low *E. coli*). Once early timepoints have been collected, timepoints will become more widely spaced, allowing initiation of additional curves.

1. Prepare a 60°C waterbath for renaturation in 0.12 M PB or a 66°C waterbath for renaturation in 0.4 M PB.
2. Denature the DNA in a boiling water bath for approximately 10 min or in a 105°C glycerol bath for 6–10 min.
3. Remove the 0 time sample and immediately plunge into a dry-ice ethanol bath to stop reassociation.
4. Label the sample and place at –20°C for storage.
5. Plunge the remaining DNA samples into a 60°C water bath for reassociation in 0.12 M PB and into a 66°C water bath for reassociation in 0.4 M PB.
6. Remove the samples at predetermined times (3.6, steps 1–5) and plunge them into a dry ice/EtOH bath to stop reassociation. Samples that are flash frozen can be stored at –20°C until HAP chromatography is performed.

16.3.8 Separation of single- and double-stranded DNA by HAP chromatography

Preparation of hydroxyapatite columns involves suspending the HAP in PB, aspirating off the 'fines', and loading syringes or minicolumns with the HAP. DNA binds to HAP *via* interactions between phosphate groups on the DNA backbone and Ca^{2+} residues on the resin. Bound single and double DNA strands are differentially eluted in PB at 60°C. At low phosphate concentrations (0.01–0.03 M PB) both double- and single-stranded DNAs are absorbed to HAP. At intermediate concentrations (0.12–0.16 M PB) single strands are eluted. Higher concentrations (0.4–0.5 M) elute double-stranded DNA. The amount of DNA reassociation is estimated by measuring the amount of DNA eluted at these different PB concentrations.

Water-jacketed HAP columns must be used to maintain the 60°C temperature required for DNA separation. Examples of apparatus are shown in Figure 16.3 and in Sambrook *et al.* (1989). An apparatus can be made fairly inexpensively by a local machine shop. The apparatus must be enclosed, house at least one column and a thermometer, and be attached to a circulating water heater to maintain a 60°C elution temperature.

1. Use a cork borer to cut circles of Whatman GF/C and 105 µm nylon mesh filters to a diameter that is slightly larger than the interior diameter of the syringe.
2. Remove the barrel from a 10 mL glass or disposable syringe and place the 105 µm nylon mesh and Whatman GF/C filters in the syringe sequentially.

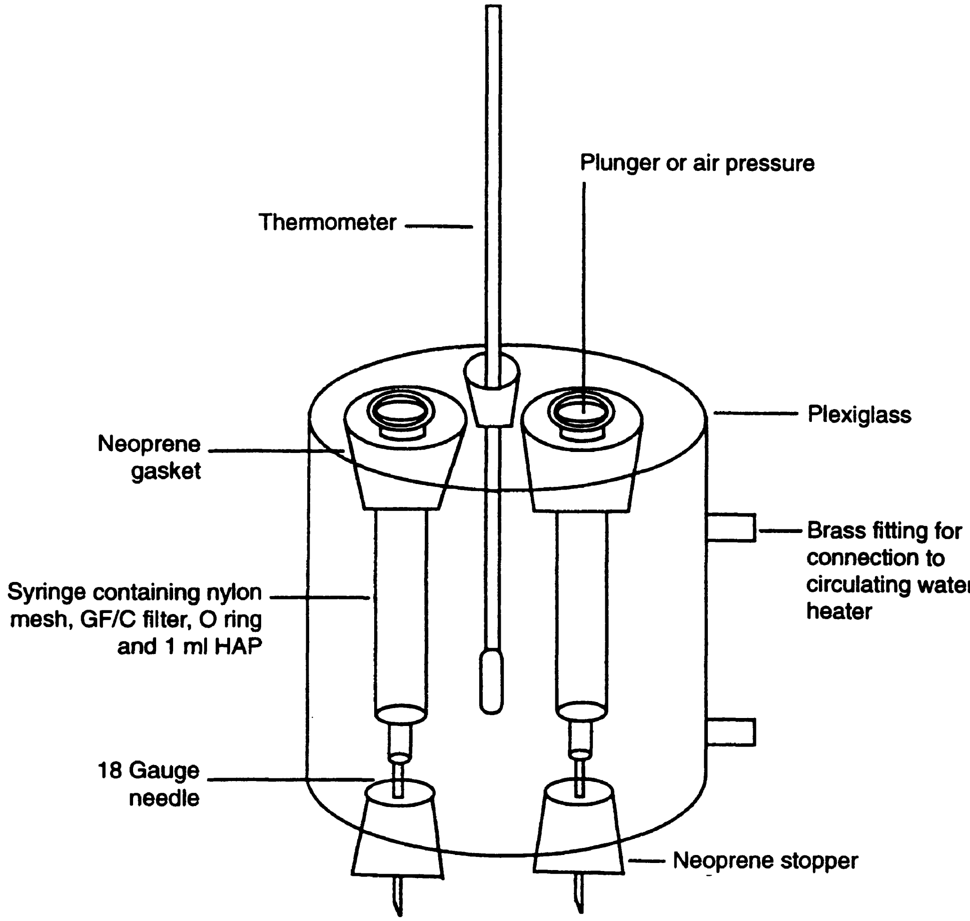


Figure 16.3 Hydroxyapatite chromatography apparatus.

3. Insert an O ring that is slightly smaller than the interior diameter of the syringe to hold the filters in place.
4. Attach an 18 G needle that has been sheared with pliers to produce a blunt end to the syringe and insert the syringe in the top of the apparatus through a neoprene gasket.
5. Seal the bottom of the apparatus by inserting a neoprene stopper of the appropriate size through the needle into the bottom of the apparatus. It is critical that the seals be tight or the apparatus will leak when the circulator is turned on.
6. Hydrate the HAP in 5–6 vol of 0.03 M PB for 1 h and aspirate off the 'fines' (HAP particulates that do not settle to the bottom).
7. Add more 0.03 M PB to the HAP, swirl, and aspirate the 'fines' again, after the HAP has settled.
8. Repeat step 7.
9. Load the HAP slurry onto the syringe and allow the excess 0.03 M PB to drain. Continue to add HAP slurry until approximately 1 mL of slurry settles in the column.
10. Add approximately 2 mL of 0.03 M PB to the top of the slurry, seal the top and the base of the column with Parafilm until use. The column should be drained immediately preceding use. Columns should only be used once.

11. Remove a frozen time point from the freezer, dilute it into 1.0 mL of 0.03 M PB, and load it on the column.
12. Stir the HAP column, allow the sample to run through, and then repeat 2–3 times to assure that most of the nucleic acid is bound. It is usually necessary to apply positive air pressure to the HAP slurry in order to quickly elute the PB and DNA from the column. Positive pressure can be gently applied using a syringe fitted with a neoprene stopper or air pressure can be applied through a needle run from a compressed air source through the stopper.
13. Equilibrate the DNA sample and buffers to 60°C before beginning the PB elutions.
14. Wash the column three times with 1.0 mL fractions of 0.03 M PB that has been preheated to 60°C.
15. Wash the column three times with 1.0 mL of 0.12 M PB at 60°C and collect the eluted single-stranded fractions into a 15 mL centrifuge tube.
16. Wash the column with three 1.0 mL fractions of 0.4 M PB preheated to 60°C, and collect the eluted fractions in a separate 15 mL tube.
17. Following elution, briefly centrifuge the single- and double-stranded fractions at 2000 rpm to pellet any residual HAP.
18. Measure the optical density of the *undiluted* 0.12 M and 0.4 M fractions directly at A_{260} and A_{320} using a PB for the blank that is identical in molarity to that of your DNA sample (i.e. 0.12 M PB blank for 0.12 M PB fractions). As renaturation proceeds you should expect to see a decrease in absorbency of single-stranded fractions and a corresponding increase in the absorbency of double-stranded fractions.
19. Subtract the A_{320} absorbency from the A_{260} to correct for nonspecific absorbency.
20. Correct for differences in absorbency of the single-stranded (1 OD = 40 μg/mL) and double-stranded (1 OD = 50 μg/mL) fractions by multiplying the A_{260} of the double-stranded DNA by 50 μg/mL and the A_{260} of the single-stranded DNA by 40 μg/mL.
21. Estimate the percentage reassociation by dividing the amount of double-stranded DNA eluted by the total amount of DNA eluted. Do not use the total amount of DNA placed into the HAP tube in the denominator because some DNA will fail to adhere to the column and some will be irreversibly bound.

16.3.9 Estimation of reassociation rates and proportion of repetitive and unique fractions

1. To fit the percentage reassociation at each C_0t value into either a three-, two- or one-parameter model follow the steps outlined in Table 16.2 for the nonlinear regression procedure in SAS. The three-parameter model is equation 16.4 listed earlier. The two-parameter model is equation 16.4 with the moderately repetitive component removed. The one-parameter model is equation 16.4 with both the highly and moderately repetitive components removed (for *E. coli* only).
2. Enter the percentage reassociation values obtained for the low, medium, and high curves. Remember to enter multiple values for time points that overlap on the three curves.
3. A specific model should be considered inappropriate when either proportions (P) or k are estimated as negative values or when the values predicted by the

Table 16.2 SAS statements used to calculate DNA reassociation kinetics. The C_0t values and amount reassociation are entered after the CARDS; statement. Note that the C_0t values are *not* entered as logarithmic values and that REASS are entered as percentages and converted to proportions

```
DATA COT;
TITLE Reassociation of short fragments in species?;
INPUT COT REASS;
REASS=REASS/100;
CARDS;
0.01 10
0.05 12
etc.
Use the following statements for a three parameter model.
PROC NLIN METHOD=MARQUARDT;
PARM PFAST=0.30 PMID=0.30 PSLOW=0.30
     KFAST = 10.0 KMID=1.00 KSLOW=0.001;
DENA=1+(KFAST*PFAST*COT);
DENB=1+(KMID*PMID*COT);
DENC=1+(KSLOW*PSLOW*COT);
FAST=PFAST/DENA;
MID=PMID/DENB;
SLOW=PSLOW/DENC;
MODEL REASS=FAST+MID+SLOW;
DER.PFAST=1/(DENA**2.);
DER.PMID=1/(DENB**2.);
DER.PSLOW=1/(DENC**2.);
DER.KFAST= -COT*PFAST*PFAST/(DENA**2.);
DER.KMID= -COT*PMID*PMID/(DENB**2.);
DER.KSLOW= -COT*PSLOW*PSLOW/(DENC**2.);
QUIT;
If instead you wish to fit your data to a two parameter model then substitute the following
statements.
PROC NLIN METHOD=MARQUARDT;
PARM PFAST=0.50 PSLOW=0.50
     KFAST = 10.0 KSLOW=0.001;
DENA=1+(KFAST*PFAST*COT);
DENB=1+(KSLOW*PSLOW*COT);
FAST=PFAST/DENA;
SLOW=PSLOW/DENB;
MODEL REASS=FAST+SLOW;
DER.PFAST=1/(DENA**2.);
DER.PSLOW=1/(DENB**2.);
DER.KFAST= -COT*PFAST*PFAST/(DENA**2.);
DER.KSLOW= -COT*PSLOW*PSLOW/(DENB**2.);
QUIT;
For estimation of k in E. coli only a single parameter model is required.
PROC NLIN METHOD=MARQUARDT;
PARM PSLOW=1 KSLOW=0.01;
DENA=1+(KSLOW*PSLOW*COT);
SLOW=PSLOW/DENA
MODEL REASS=SLOW;
DER.PSLOW=1/(DENA**2.);
DER.KSLOW= -COT*PSLOW*PSLOW/(DENA**2.);
QUIT;
```

COT, the Cot value at which reassociation determination was made; REASS, the proportion of DNA that was reassociated at COT; PFAST, proportion of the genome comprised of highly repetitive sequences; PMID, proportion of the genome comprised of moderately repetitive sequences; PSLOW, proportion of the genome comprised of unique sequences; KFAST, reassociation rate of highly repetitive sequences; KMID, reassociation rate of moderately repetitive sequences; KSLOW, reassociation rate of unique sequences.

derived model do not fit the actual data. For example, small genomes with long period interspersion do not fit a three parameter model and a two parameter model should be used instead.

4. The $C_0t_{1/2}$ for each component is estimated as (1/*k*). Genome size, interspersion patterns, and repeat frequencies (complexity) are calculated from equations 16.5 to 16.7) in the introduction.

16.3.10 An alternative method for C_0t curve analysis when the amount of arthropod DNA is limiting

When the amount of DNA is limited, an alternative method that employs ^{3}H-radiolabelled tracer DNA can be used to quantify reassociation. Because of the sensitivity of the radiolabel, this method requires only 300–500 μg of DNA per curve. The major drawback of this method is the potential for generating a population of radiolabelled tracer fragments that differ in size from unlabelled DNA fragments. Because labelling DNA by nick translation introduces nicks to incorporate nucleotides, this procedure results in a successive decrease in the fragment length of the DNA as the reaction proceeds. Therefore, it is critical that you first optimize nick translation conditions with both *E. coli* and calf thymus DNA before proceeding with your arthropod samples. Reduction in fragment length of the tracer will affect the reassociation kinetics of the DNA. Sambrook *et al.* (1989) also provides a thorough discussion of procedures for optimizing nick translation. **You will be working with ^{3}H-dCTP throughout most of this protocol. Your laboratory must be authorized to use ^{3}H-dCTP and you must follow proper handling and disposal procedures.**

1. Isolate genomic DNA as outlined in Section 16.3.1.
2. Follow the manufacturers instructions for radiolabelling 1 μg of DNA using intact *E. coli* and calf thymus DNAs.
3. Remove unincorporated nucleotides by Sephadex G-50 spin column chromatography (Sambrook *et al.*, 1989) and precipitate with 1/10 vol 3 M NaOAC and 2.5 vol of EtOH. Resuspend in a small volume (10–20 μL), add gel dye, and electrophorese with nonradiolabelled intact and sonicated DNAs, and with λ *Hin*dIII and 100 bp ladders. This will allow you to determine the degree of reduction in fragment length generated by nick translation.
4. If the fragment length is too small (< 1–10 kb) for large fragments, try reducing the length of the nick translation reaction, reducing the temperature, reducing the amount of DNase I in your nick translation (Notes), or some combination of all three.
5. If the fragment length is too large (>1–10 kb for large fragments or >100–1000 bp for small fragments) try briefly sonicating the DNA to reduce the fragment length.
6. Once you have produced populations of short (0.1–1 kb) and large (0.5–10 kb) fragments whose average lengths are slightly larger than 0.3–0.5 kb (short) or 2–4 kb (long), perform several nick translations of arthropod and *E. coli* DNA simultaneously (1 μg of DNA × 6) and remove unincorporated nucleotides as outlined in step 3.
7. Pool the nick translations from one species, remove a 1 μL aliquot, and measure the ^{3}H-dCTP incorporation in a scintillation counter. A specific activity between 10^5-10^6 cpm/μg should suffice for C_0t analysis.

8. To produce a population of unlabelled DNA fragments in the same size range, scale down sonication conditions outlined in Section 16.3.2 by diluting DNA samples to ~250 μg/mL and sonicate 2 mL aliquots in a 6 mL (12 × 27 mm tube) as outlined in Section 16.3.2. All earlier precautions (Section 16.3.2) apply.
9. When unlabelled DNA fragments appear to be in the same size range as the radiolabelled tracer, ethanol precipitate a 1 μg aliquot of radiolabelled tracer DNA as outlined in step 3 and electrophorese on a 1.2% (short) or 0.7% (long) gel along side a 1 μg aliquot of unlabelled DNA and an appropriate molecular weight marker.
10. If necessary, briefly shear the remaining unlabelled DNA population to reduce the fragment size to a comparable fragment length and repeat step 7.
11. Approximately 0.5 μg (low C_0t), ~2.5μg (medium C_0t), and 5–10 μg (high C_0t values) of DNA will be required per time point. Refer to Section 16.3.6 for the number of time points required to execute each C_0t curve.
12. Mix unlabelled DNA with radiolabelled tracer DNA (approximately 10–25 × 10^3 cpm/time point) and precipitate with 1/10 vol 3 M NaOAC and 2.5 vol 100% EtOH. It is important that the radiolabelled tracer and unlabelled DNA be mixed prior to the final measurement of the DNA concentration to accurately calculate C_0t values and time points.
13. Follow the steps outlined in Section 16.3.4.
14. Dispense the DNA in 2–5 μL aliquots in 10 μL microcapillary tubes using a micropipettor and then heat seal the tube by holding the microcapillary tube over a flame and using forceps to 'pull' the excess glass away. Mineral oil is not necessary since this is a sealed system.
15. Follow the steps outlined in Sections 16.3.6 and 16.3.7.
16. Open microcapillary tubes using a file (*wear safety goggles*) and dispense the contents into 1 mL 0.03 M PB using a microcapillary pipette filler. Follow the steps outlined in section 3.8 for separation of double- and single-stranded DNA. There will not be enough DNA to measure the absorbency values.
17. Mix the samples well and aliquot 3 mL of the double- and single-stranded fractions into a scintillation vial containing 17 mL of a scintillation liquid recommended for counting aqueous samples (i.e. Scintiverse II, Fisher).
18. Count double- and single-stranded samples for each time point. Samples are generally counted for 5 min using a programme designed to detect ^3H. It is important to include a 3 mL blank of 0.12 M PB and a 3 mL blank of 0.4 M PB as controls for background and/or quenching. Subtract the cpm of the blanks from the cpm of each sample before estimating reassociation.
19. Percentage reassociation is estimated as the amount of double-stranded DNA eluted divided by the total amount of DNA eluted.
20. Follow the steps outlined in Section 16.3.9 to estimate genome size and the pattern of repetitive DNA interspersion.

NOTES

1. Several seminal papers provide in-depth discussions of various parameters involved in DNA reassociation kinetic analysis (Britten and Kohne, 1968; Wetmur and Davidson, 1968; Britten *et al.*, 1974; Davidson *et al.*, 1975). We also

encourage you to read the discussion of renaturation kinetics in *Gene IV* (Lewin, 1980).

2. The purity of the DNA used in reassociation kinetics is critical since contaminating proteins and metal ions can substantially alter the kinetics of reassociation. Methods that employ caesium chloride gradients and ultracentrifugation result in efficient separation of proteins, RNA, and DNA. Following isolation of DNA, the ratio of the optical density at 260_{nm} and 280_{nm} should be 1.8. Lower ratios are indicative of contamination with proteins or ions that will interfere with reassociation. Higher values suggest RNA contamination that can also bias estimates of unique sequences.
3. 0.3% agarose gels should be poured over a very thin 1% agarose base. The gel comb should be elevated so that, when poured, the wells will consist only of 0.3% agarose. Otherwise, the DNA will run at two different mobilities. Do not overload the marker lane (no more than 200 ng of standard) or there will be significant smearing of the DNA.
4. Because it is difficult to produce long DNA that is not too short to be used reliably for interspersion determination with sonication, we developed an alternative approach utilizing partial *Mbo*I (or *Sau*3AI) digestion when DNA is limiting. This approach is similar to that utilized when generating random DNA fragments for construction of genomic DNA libraries. Details of the procedure can be found in Palmer *et al.* (1994).
5. Small DNA fragments should appear as a smear between approximately 0.1–1 kb and large fragments should appear as a smear between approximately 0.5–10 kb. It is critical that the DNA be sheared sufficiently (<15 kb) because large fragments can form concatamers during renaturation that will bind irreversibly to the HAP columns. Calf thymus DNA is a useful standard for determining optimum sonication conditions for arthropod genomic DNA. If your large DNA fragment population contains a significant population of DNA fragments >10 kb (similar to the distinct band seen in lane 1, Figure 16.2) it is imperative that these larger fragments be reduced in size before performing C_0t analysis. Brief sonication will often remove them without significantly reducing the overall fragment size.
6. If you see a decrease in absorbency in both single- and double-stranded fractions, such that the percentage of double-stranded DNA is not increasing with time, measure the absorbency of the HAP from your column using HAP in 0.4 M PB as a blank. If the absorbency of the HAP in the column is greater than the HAP in the blank then DNA has been retained on the column. This can be avoided by preparing a population of long DNA that doesn't contain fragments larger than ~10 kb.
7. Phosphate salts precipitate in ethanol. Once DNA has been resuspended in PB, the PB must be extracted through dialysis. This results in substantial loss of DNA. Make sure that your DNA is the right size and that you know your approximate yield before resuspending in PB!
8. Before running your samples over HAP columns, first run control columns with untreated (double-stranded) and heat denatured (single-stranded) *E. coli* DNA to assure that ~95% of the double-stranded DNA elutes in 0.4 M PB and ~95% of the single-stranded DNA elutes in 0.12 M PB fractions.
9. In 0.12 M PB, the relative reassociation rate (γ) is 1.0. The equivalent C_0t for higher PB concentrations is obtained by multiplying the C_0t in 0.12 M PB by γ for alter-

native salt concentrations (the γ for 0.4 M PB is 4.049). A table listing relative reassociation (γ) versus salt concentration can be found in Britten *et al.* (1974).

10. As a general rule, renaturation should proceed one log past $C_0t_{1/2}$ to obtain a complete curve (i.e. if $C_0t_{1/2}$ is 100, then renaturation is measured to C_0t 1000). At least 90% of the DNA should be renatured to achieve a representative C_0t curve.
11. To obtain a more accurate time point for lower C_0t values divide the hours into minutes and/or seconds.
12. Low C_0t values for *E. coli* will be derived from the analysis of the DNA at low concentration while the medium C_0t values will be estimated from the medium concentration. The reassociation curve will be a composite of two different reassociation reactions; one in the low concentration solution and another in the medium concentration solution. Late C_0t points in the low concentration solution should equal or overlap early points in the middle C_0t. It is important to check reproducibility of rates among reassociation conditions to assure continuity between low and medium curves. Once the *E. coli* reassociation experiment is completed, the derived curve and $C_0t_{1/2}$ should be compared with published values for *E. coli* (Lewin, 1980). If there are not large discrepancies with published work then proceed to work on arthropod DNA. Note that the value of k is affected by many variables in the experiment and while exact values will not be obtained they should be in the range of 1–4 M·s/L.
13. Late C_0t values for arthropod DNA in the low concentration solution must overlap or equal early C_0t values in the medium concentration solution. Similarly plan for late C_0t values in the medium concentration to overlap early C_0t in the high concentration solution. The low and medium C_0t values for an arthropod can be completed in 1–2 days, but the high C_0t values will require 2–3 days of reassociation. Remember also, that a second C_0t curve for long fragments must be derived to describe the pattern of repeat interspersion.
14. *E. coli* DNA polymerase I adds nucleotide residues to the 3′-hydroxy termini created when one strand of a double-stranded DNA molecule is nicked by DNase I. Because the enzyme also contains a 5′ to 3′ exonuclease activity that removes nucleotides from the 5′ side of the nick, radiolabelled nucleotides can be incorporated on both sides of the nick as the enzyme moves along the DNA. If DNase I concentrations become too high, nicking of the template can cause a substantial reduction in fragment length. Therefore, it may be necessary to purchase DNase I and DNA polymerase I separately and to titrate the amount of DNase I added to the nick translation to obtain optimum nick translation of your DNA. You may also want to consider a combination of nick translation and sonication to produce a population of tracer DNA for C_0t analysis.
15. Because some fraction of DNA will renature instantaneously while some will fail to renature during the course of the C_0t curve, you will not obtain single-or double-stranded values of 100%. In general, approximately 95% of the DNA will be single-stranded at time O and approximately 90% should be double-stranded at the last time point.

CONCLUSION

The most critical step in performing C_0t curve analysis is the preparation and fragmentation of your DNA. If adequate care is taken to produce populations of DNA

fragments that do not contain DNA that is too large (>10–15 kb), or too small (<200 bp), then C_0t curve analysis can often be performed once, using the steps outlined above. Arthropods, and insects in particular, comprise the most specious taxon. Our understanding of genome evolution could benefit significantly from a more thorough examination of genome size and organization in a variety of arthropods.

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CHAPTER SEVENTEEN

Constructing and screening lambda libraries

Margaret A. Hughes

17.1 INTRODUCTION

A genomic library is a representative collection of DNA fragments from a particular organism inserted into vector molecules which are capable of self-propagation in a suitable host cell. It provides a convenient facility for the selection of specific DNA sequences including whole genes (plus the flanking regions), gene fragments, small gene clusters, repetitive sequences as well as allowing large scale mapping experiments *via* 'genome walking'. Genomic expression libraries can also be constructed where intron sequences constitute a relatively small proportion of the coding regions.

A library can be constructed using different vector systems based on either bacteriophage lambda, cosmids or plasmids. In general, an ideal library should meet the following criteria:

1. It must contain DNA sequences representative of the entire genome in a stable form.
2. There must be a manageable number of overlapping clones which are large enough to contain whole genes but small enough to facilitate mapping by restriction enzyme analysis.
3. It should be both easy to construct from small amounts of starting material and easy to screen by hybridization.
4. It should be possible to amplify the library without loss or misrepresentation of sequences and to store it for long periods of time without a significant loss of titre.

We can come close to these ideals by using bacteriophage λ as a cloning vector. The cloning efficiency obtained with λ is relatively high and the manipulation of >100 000 clones needed to ensure a complete library of genomic sequences is convenient.

Figure 17.1 shows a diagrammatic scheme for cloning insect DNA into a phage λ vector. Only 60% of the DNA of wild type λ (48.6 kb) is necessary for its lytic cycle. These essential genes are contained on the left and right arms and the nonessential genes are contained in the central 'stuffer' fragment. It is this fragment which is replaced for the propagation of non λ DNA fragments of a suitable size. This size restriction is because only λ DNA molecules falling in the range of 40–52 kb can be

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packaged into phage particles and only then if it is capable of forming concatemers (Hohn and Hohn, 1974).

In Figure 17.1, it can be seen that λ DNA has single stranded complementary ends known as *cos* ends. These ends are annealed and ligated prior to the removal of the stuffer fragment (Step 1) thereby ensuring that concatemers are formed later on after the ligation of the insert DNA. This condition is the ideal format of the DNA for the *in vitro* packaging reaction.

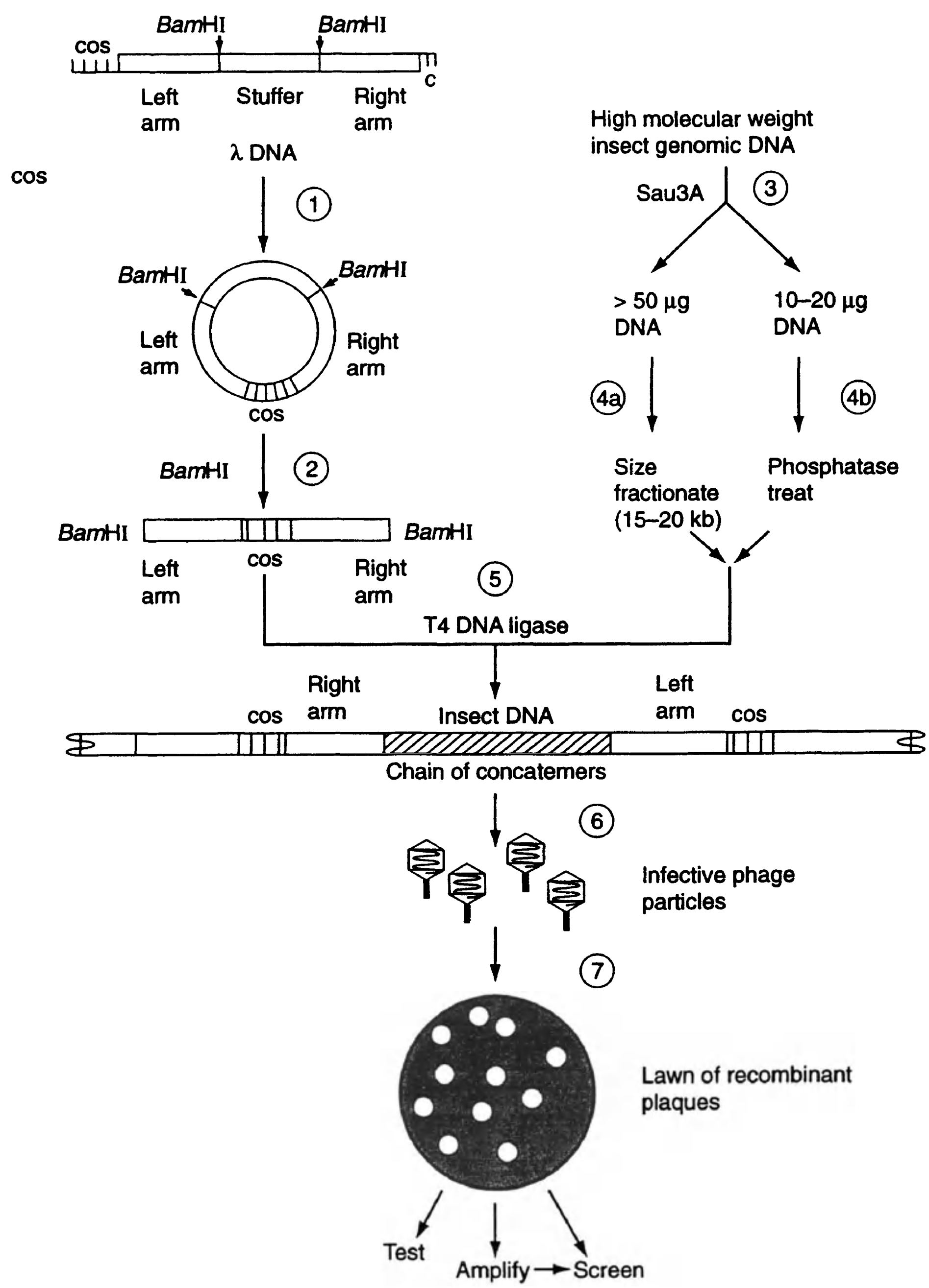


Figure 17.1 Diagrammatic scheme for cloning insect DNA into a phage λ vector. Each step is described in the text.

Once the λ DNA has been cut with a suitable restriction enzyme (Step 2), the stuffer fragment must be physically removed to prevent its religation which would otherwise result in a high background of nonrecombinants. If the vector system used is able to minimize background by genetic selection e.g. *Spi* phenotype (Zissler *et al.*, 1971), a purification step at this stage is not essential.

The insect genomic DNA used must be of a very high molecular weight and ideally purified using mild procedures. To generate a complete library, it is necessary to obtain a near random collection of fragments as possible within a size range suitable for the λ vector chosen. This fragmentation is most commonly achieved by setting up a range of partial digestions of the DNA with one or two frequently cutting enzymes such as *Sau*3A (Step 3). These digestions are mixed, hopefully to give a set of overlapping fragments. If the amount of starting material is large i.e. >50 μg (Step 4a), fragments of the desired size are selected by velocity gradient centrifugation. However, if only small amounts of material are available, this fractionation step is not feasible and one must rely on the packaging system to size select recombinant molecules of the correct total size after ligation. Multiple inserts can be prevented by treating the partially digested DNA with phosphatase (Step 4b). However, it must be remembered that partial digestion may not yield a truly random mixture for a given size range and this fact needs to be taken into account when screening for a particular recombinant i.e. more clones than expected may need to be screened.

Lambda arms and the insert DNA are joined together by T_4 DNA ligase (Step 5) to form long concatemeric chains which are subsequently packaged into infectious particles (Step 6) using *in vitro* packaging systems. As already discussed, only λ DNA molecules falling in the size range of 40–52 kb will be packaged into phage heads with high efficiency. These particles are used to infect a suitable *E.coli* host (Step 7) resulting in the formation of a lawn of recombinant plaques.

Once a library is made it can be screened for the presence of specific recombinants as outlined in Figure 17.2. The number of recombinants to be screened can be calculated using the formula of Clarke and Carbon (1976) (Chapter 18 gives details on cosmid libraries). Bacteriophage are plated at an appropriate density and an imprint of the pattern of plaques is obtained by gently overlaying the lawn with a nitrocellulose filter. This is treated with alkali to liberate the DNA which is subsequently fixed by baking. The filter is then incubated in the presence of the specific DNA probe which will hybridize to any complementary sequences that may be present. After washing away the excess probe, the filters are exposed to autoradiographic film. If a positive signal is obtained, the clone can be picked by alignment to the original plate.

This chapter will describe the technical procedures required to carry out all the steps outlined above for the construction and screening of a λ genomic library. However, please note that the details of restriction enzymes, vectors and host cells have been omitted since this depends on the system being used and the reader is advised to follow the manufacturer's guidelines. Kaiser and Murray (1985) and Sambrook *et al.* (1989) are also useful sources of information.

17.2 MATERIALS

17.2.1 General points

1. All plasticware should be sterilized by autoclaving. Reagents and bacteriological media should be sterilized by autoclaving. Filter-sterilize if the buffer contains a labile component e.g. DTT.

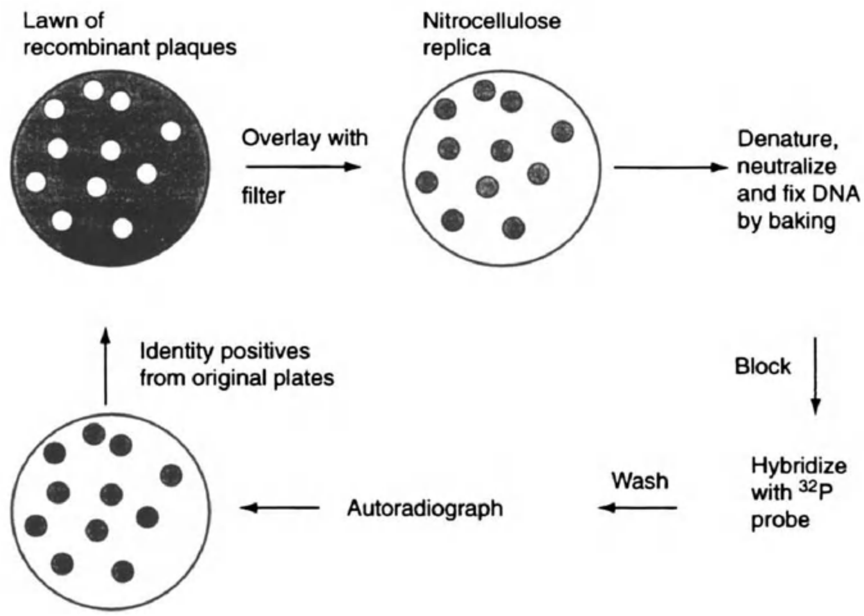


Figure 17.2 Diagrammatic representation of the protocol for screening genomic libraries in λ.

2. Sterile microbiological procedures are necessary when handling bacteria and infectious phage. Before starting work, make sure that the laboratory is appropriately registered by local authorities for carrying out recombinant work.
3. Gels are made up with standard agarose in 1xTAE buffer (10xTAE is 0.4 M Tris-acetate, 0.01 M EDTA pH 7.7). The agarose is melted by boiling and allowed to cool ~50°C before pouring into a suitable gel tray for horizontal electrophoresis. Bands can be visualized on a UV transilluminator after staining with ethidium bromide at 0.5 μg/mL. Ethidium bromide is made as a stock solution of 10 mg/mL in water and must be handled with care as it is a potential carcinogen.
4. Labelling and hybridization experiments with ^{32}P involve radiation hazards. Therefore handling and disposal must be in accordance with local rules.

17.2.2 Buffers

1. TE: 10 mM Tris-HCl, pH 7.5, 1 mM EDTA.
2. 20×SSC (per L): 175.3 g NaCl, 88.2 g sodium citrate.
3. 3 M sodium acetate, pH 5.2.
4. L agar (per L): 10 g bacto-tryptone, 5 g yeast extract, 10 g NaCl and 15 g agar. This is poured into Petri dishes after sterilizing. Top agar or top agarose for pouring phage onto plates is the same base except that it contains only 7 g/L agar or agarose respectively.
5. Ligation buffer (10×): 0.5 M Tris-HCl, pH 7.6, 100 mM $MgCl_2$, 100 mM dithiothreitol, 500 μg/mL bovine serum albumin (fraction V). Filter-sterilize and store in small aliquots at –20°C. ATP should be added before use so that the final concentration of ATP in the reaction is 0.5–1 mM.

6. SM buffer (per L): 50 mL 1 M Tris-HCl, pH 7.5, 2 g $MgSO_4.7H_2O$, 5.8 g NaCl, 5 mL 2% gelatin.
7. Denaturing buffer: 1.5 M NaCl, 0.5 M NaOH.
8. Neutralizing buffer: 1.5 M NaCl, 0.5 M Tris-HCl, pH 7.5.
9. Hybridization buffer (per 100 mL): 50 mL deionized formamide, 25 mL 20×SSC, 1 mL denatured herring sperm DNA (5 mg/mL), 1 mL yeast tRNA (10 mg/mL), 1 mL polyA RNA (1 mg/mL), 1 mL 10% SDS, 1 mL 100× Denhardt's solution and water to 100 mL.
10. 100× Denhardt's solution (per L): 20 g ficoll, 20 g polyvinylpyrrolidone, 20 g bovine serum albumen (fraction V).
11. For salt gradients, make up separately a 5% NaCl solution and a 30% NaCl solution in 10 mM Tris-HCl, pH 7.5, 2 mM EDTA.

17.3 METHODS

17.3.1 The isolation of insect DNA

Details for the isolation of genomic DNA from insects can be found in Chapter 14. It is essential that the DNA is of a very high molecular weight particularly if random fragments are to be generated by partial digestion with restriction enzymes.

The integrity of the DNA should be checked on a 0.5% agarose gel with appropriate size markers e.g. intact λ DNA. DNA with a high molecular weight will run as a relatively tight band behind the λ DNA with little or no smearing in the lower areas of the gel.

17.3.2 Partial digestion of genomic DNA

To generate random fragments of the desired size (e.g. 15–20 kb), optimal conditions are first established on a small scale for a particular batch of DNA.

1. Dilute 10 μg of genomic DNA with 10 μL of 10× restriction buffer and sterile water to a final volume of 100 μL. DNA must be homogeneously dispersed in solution otherwise the rate of digestion cannot be controlled (Note 1).
2. Dispense 20 μL of DNA solution to a microfuge tube (tube 1) and 10 μL to a further eight tubes (tubes 2–9) using a pipette tip with a cut off end. Stand the tubes on ice.
3. Add 2 U of an appropriate restriction enzyme to tube 1 (1 U/μg). Gently mix with a sealed glass capillary. Using a fresh pipette tip, transfer 10 μL of the reaction to tube 2 (0.5 U/μg) and mix as before. Continue transferring the reaction to successive tubes up to tube 8. Discard 10 μL from this tube. Do not add anything to tube 9 (no enzyme control).
4. Place the tubes in a 37°C water bath and incubate for 1 h. Stop the reaction by heating the tubes at 70°C for 15 min.
5. Run the samples on a 0.5% agarose gel at a low voltage (2 V/cm) at 4°C in order to prevent smearing. Include suitable size markers e.g. λ DNA cut with *Hin*dIII.
6. Stain the gel with ethidium bromide (0.5 μg/mL) for 30 min followed by destaining in water. Photograph the gel, taking care not to overexpose the film.

7. Determine the enzyme concentration which produces the maximum fluorescence in the desired size range e.g. 15–20 kb for many replacement vectors. Using half that enzyme concentration will produce the maximum number of molecules of that size (if problems are encountered with digestion, see Note 2).

17.3.3 Preparation of insert DNA

Having established the optimal conditions, large scale digests can be set up providing that there is sufficient DNA. Fragments are then separated on a salt gradient. However, if only small quantities of DNA are available, this will not be possible and a compromise is suggested (Note 3).

1. Place 50–100 μg DNA into three separate tubes and dilute with restriction buffer and water to the same concentration as used in the pilot reaction above. Prewarm the tubes to 37°C.
2. Place the optimum amount of enzyme in one of the tubes and concentrations of enzyme which stagger either side of this optimum into the other two tubes. This will help minimize a misrepresentation of fragments containing an abnormal distribution of restriction sites. Incubate at 37°C for 1 h and stop the reaction at 70°C as before.
3. Place the tubes on ice. Analyse an aliquot of each digest (0.5 μg) on a 0.5% agarose gel (run at 4°C as before) to check that the correct size distribution has been obtained.
4. Pool the samples and gently extract twice with an equal volume of phenol:chloroform (1:1 v/v). Centrifuge and recover the aqueous phase.
5. Adjust NaCl concentration to 0.3 M and add 2 vol of ethanol. Place on ice for 10 min.
6. Recover the DNA by centrifugation at 12 000 **g** for 10 min at 4°C. Wash the pellet twice with 70% ethanol and spin as before.
7. Dry the pellet *in vacuo*. Resuspend DNA in 200–400 μL TE. If necessary this can be speeded up by heating the sample at 65°C for 10 min.

17.3.4 Size fractionation on NaCl gradient

DNA fragments can be fractionated on velocity gradients through either sucrose or salt to concentrate fragments of the required size for cloning. The latter method is described here.

1. Pour a 5–30% (w/v) NaCl gradient in a 12.5 mL centrifuge tube e.g. Beckman SW41. The salt solutions are made up in 10 mM Tris-HCl pH 7.5, 2 mM EDTA.
2. Layer the DNA sample (150 μg in 200 μL) onto the top of the gradient. Do not exceed this amount of DNA as overloading will result in poor resolution.
3. Centrifuge at 37 000 rpm for 4.5 h at RT (Beckman SW41).
4. Collect 300–400 μL fractions into sterile microfuge tubes.
5. Take 1/10–1/20 aliquots from each fraction and dilute it with 3–4 vol of TE. Store the remainder of the sample at –20°C for use later.
6. Run the aliquots on a 0.3% agarose gel at 4°C with suitable size markers which have been adjusted to a comparable salt concentration as the samples so that the mobility is relative.

7. After detecting which of the fractions contain the desired size range of fragment e.g. 15–20 kb, pool the appropriate samples, dilute with an equal volume of sterile water and add 2 vol of ethanol. Mix and leave overnight at –20°C.
8. Collect DNA by centrifugation at 12 000 g for 15 min at 4°C. Wash the pellet twice with 70% ethanol and dry *in vacuo.*
9. Resuspend the DNA to an estimated concentration of 300–500 μg/mL in TE. Check the concentration and size by running an aliquot on a gel. Store the inserts at –20°C.

17.3.5 Preparation of vector arms

When using replacement λ vectors for the construction of genomic libraries, the central fragment is removed by digestion with an appropriate restriction enzyme. The fragment is then purified from the arms by velocity gradient centrifugation. However, if genetic selection is available against nonrecombinant phage, this fractionation step is not necessary and the vector can be used directly after cleavage and inactivation of the enzyme.

Before preparing the arms, it is a good idea to check that the vector has the expected restriction sites and that the intact vector gives a high packaging efficiency with extracts of known efficiency.

The *cos* ends of λ can be annealed and ligated prior to digestion to ensure that concatemers are formed after ligation to the insert. If preferred, the vector DNA can be restricted first and annealed afterwards (Note 4).

1. Incubate 60 μg λDNA for 1 h at 42°C in 170 μL 0.1 M Tris-HCl pH 7.5, 10 mM $MgCl_2$. Add 20 μL 10× ligation buffer and 0.2-0.5 U ligase/μg DNA and incubate 1–2 h at 37°C. If the DNA is not to be size fractionated, it is possible to start with a smaller amount of DNA e.g. 10–20 μg.
2. Gently extract with an equal volume of phenol:chloroform (1:1 v/v), recover the aqueous phase after centrifugation and precipitate the DNA by the addition of 1/10 volume of 3 M sodium acetate pH 5.2 and 2 vol of ethanol.
3. Recover the DNA by centrifugation and after briefly drying, resuspend it in a suitable volume of 1× restriction buffer. Add a 2–3 fold excess of the appropriate restriction enzyme. Incubate at 37°C for 1–2 h.
4. Check an aliquot of the DNA (0.5 μg) on a 0.5% agarose gel. Keep the remainder of the reaction on ice. Add more enzyme to this if necessary and continue incubation if the digestion is found to be incomplete.
5. Inactivate the enzyme by extracting once with an equal volume of phenol:chloroform (1:1 v/v) and recover DNA by precipitation with 1/10 volume 3 M sodium acetate pH 5.2 and 2 vol of ethanol.
6. At this stage there are two alternatives depending on the nature of the vector. Either: (i) the stuffer fragment is removed by velocity gradient centrifugation (Section 17.3.4). Load 50 μg DNA onto a 5–30% NaCl gradient. Check aliquots of the fractions on a 0.5% agarose gel. Recover the arms and resuspend in TE to a final concentration of 300–500 μg/mL. Store in aliquots of 1–5 μg at –20°C. Alternatively: (ii) for vectors which utilize genetic selection against nonrecombinants e.g. EMBL series, the DNA can be used directly for ligation. If desired, the arms can be dephosphorylated with alkaline phosphatase (Sambrook *et al.*, 1989). In this case, do not use insert which has been similarly treated with phosphatase (Note 3).

17.3.6 Ligation

It is necessary to determine the optimum concentration of λ vector and insert DNA for the ligation reaction. Although this can be theoretically calculated, in practice a percentage of the DNA molecules will have damaged ends or other problems. Also the concentration of insert may be wrongly estimated. Trial ligations are therefore first set up to establish the ideal ratio of vector arms to insert. Commercial packaging extracts are convenient and recommended unless time allows for making them oneself. Various methods for this procedure can be found in Sambrook *et al.*, 1989.

1. Set up a series of 10 μL ligation reactions containing 1 μg λ arms (final concentration is 100 ng/mL), 1 μL 10× ligation buffer, 0.5–1 U T_4 DNA ligase and varying amounts of insert e.g. 15, 30, 60 and 120 ng (Note 5). Include a 'no insert' control.
2. Incubate at 16°C for 4 h to overnight.
3. Package the ligation reactions into phage particles using extracts of known efficiency (previously tested with intact λ wild type DNA).
4. Infect a suitable host bacteria and plate out a series of titrations.

For vector arms (30 kb) at 100 ng/mL, an equimolarity of insert (20 kb) is 67 μg/mL. The ligation containing the optimum ratio of arms:insert should yield at least 10^6–10^7 recombinants/μg λ arms when using commercial packaging extracts. Background levels of nonrecombinants must also be accessed by appropriate means and taken into account.

Having established these parameters, further ligations can be set up using the same optimal conditions and packaged so that at least a genome equivalent of recombinants is obtained. Five to ten genome equivalents are preferable to maximize the possibility of identifying the sequences of interest.

17.3.7 Amplification

When a library is constructed, a choice has to be made as to whether to screen the primary library directly or to amplify it. The advantages of amplification are obvious in that there is then an unlimited supply – there is a library to go back to for each experiment! It is also stable over a long period of time. However, there can be a problem in that different recombinants will grow at different rates resulting in a misrepresentation of the recombinants in the amplified library. Repeat isolates of the same clone will unavoidably be characterized and several genome equivalents may have to be screened to find a variant particularly in the case of gene families.

The primary library should therefore be used if there is enough starting material and a limited number of screens are to be made. It should also be used for the isolation of poorly growing recombinants. The primary library is best stored as a ligation reaction at –20°C and packaged when needed since the packaged particles have a limited shelf life.

However, having an amplified library to hand is convenient and the method below describes how to achieve this. Amplify at least one genome equivalent although, again, 5–10 genome equivalents are optimal.

1. Mix batches of 10 000–20 000 phage (in a volume of 50 μL or less) with 200 μL of the appropriate plating cells. Adsorb for 20 min at 37°C.
2. Add 6.5 mL top agar (cooled to 50°C) and gently mix by rolling. Quickly pour the mixture onto a fresh 150 mm agar plate.

3. After the top agar has set, invert the plate and incubate at 37°C for 8–10 h. Do not allow the phage to grow so big that they touch each other (Note 5).
4. Overlay the lawn with 12 mL SM buffer and leave overnight at 4°C on a level surface.
5. Recover the SM buffer and place it into a polypropylene tube. Rinse the plate with 4 mL SM and add to the tube. Add chloroform to a final concentration of 5% (v/v) and incubate at RT.
6. Centrifuge at 4000 g for 5 min.
7. Recover the supernatant and aliquot into sterile glass Bijou bottles. Add chloroform to a final concentration of 0.3% (v/v). Store at 4°C.
8. Check the titre of the library. This should be fairly stable for several years but should be checked from time to time.

17.3.8 Checking the quality of the library

The following checks should be made on the completed library.

1. Screen the library with a sequence of known representation in the genomic DNA. Any variation in the expected number of clones may indicate that the library is not truly representative of the starting DNA.
2. Prepare DNA from the library and cleave this and the starting DNA with a given restriction enzyme. After electrophoresis, blot the gel and probe the blot with a given sequence. This will indicate the relative representation of the sequence in the two DNAs as well as showing that the particular sequence is present in the library.
3. Isolate DNA from random clones to check the size distribution of inserts.

17.3.9 Preparation of nitrocellulose lifts

After a library has been prepared, it is usually desirable to isolate specific recombinants from the population. The most commonly used method involves screening of plaques by hybridization with a ^{32}P labelled probe (Benton and Davis, 1977). The number of recombinants that are needed for screening can be calculated as previously discussed.

1. Plate the λ library in top agarose (Note 7) onto agar plates which are 1–2 days old and free from condensation. The plaque density may be as high as semi-confluent i.e. the plaques may be just touching but should still be individually discernible. For a 90 mm dish, this is about 15 000 plaques and for a 150 mm dish, about 50 000 plaques.
2. After growth, chill the plates at 4°C for about 1 h to allow the top agarose to harden.
3. Prewet the nitrocellulose filters in 3×SSC and blot on Whatman 3MM paper. Wrap the filters in cling film and sterilize them by exposing to UV for 30 s on each side.
4. Place a filter onto the agar plate by gently bending and lowering the centre of it. Asymmetrically mark both the filter and the bottom of the Petri dish with a waterproof pen. Alternatively, pierce the filter and the agar plate with a hypodermic needle previously dipped in Indian ink.

5. After 1 min, peel off the filter with blunt ended forceps. If the agar starts to lift, gently lower the filter and start again at another point.
6. Place the filter, plaque side up, onto Whatman 3MM paper soaked in denaturing buffer and leave for 5 min.
7. Transfer the filter into two successive dishes of neutralizing buffer, leaving it for 1 min in each. Air-dry on 3MM paper.
8. Duplicate filters can be taken in the same manner as above but leaving them in contact with the agar for slightly longer each time.
9. Bake the filters at 80°C for 2 h in between sheets of 3MM paper.
10. Store the agar plates at 4°C until the screening is complete.

17.3.10 Hybridization of the filters to ^{32}P labelled probe

Methods for labelling various DNA probes include random priming (Feinberg and Vogelstein, 1983), nick translation (Rigby *et al.*, 1977) and end labelling (Bollum, 1974; Maxam and Gilbert, 1977). Many kits are available on the market to carry out these protocols. If the probe is double-stranded, it is crucial that this is denatured by boiling for 5–10 min before it is added to the hybridization buffer.

1. Place the filters in a tray containing 0.1 M NaOH and agitate for 5 min. Rinse them in water before transferring them to a tray containing 0.1 M Tris-HCl pH 7.5 and wash for 5 min.
2. Place the nitrocellulose filters into a suitable container for hybridization e.g. a heat-sealable bag, a plastic sandwich box with an airtight lid or a specialized glass cylinder for a hybridization oven.
3. Add just sufficient hybridization solution to cover the filters so that they do not stick together and can move freely. When using plastic bags, ensure that all the air bubbles are removed before sealing it. Agitate the filters at 42°C for at least 4–6 h. If preferred, this prehybridization step can be left overnight.
4. Remove the exhausted hybridization solution and replace with fresh solution containing the denatured probe. As a general rule, allow 10^5–10^6 cpm/mL of hybridization solution for a DNA probe of specific activity of 5×10^7–5×10^8 cpm/μg. Allow 50–100 μL solution/cm^2 nitrocellulose. Incubate at 42°C overnight with agitation.
5. Carefully remove the probe and place the filters in a tray containing 2×SSC and 0.1% SDS at RT. Agitate for 5 min and pour the washing solution away. Repeat this washing step. At no stage during washing should the filters be allowed to dry out as this will cause background on the final autoradiograph.
6. Continue to wash the filters for 1 h at the required stringency (Chapter 20) and monitor them with a Geiger counter (Note 8, which describes in more detail the factors to consider during the washing procedure).
7. When the desired level of counts has been obtained, blot the excess wash solution from the filters with Whatman 3MM paper. Place them onto a backing card previously covered with cling film and cover the filters with another piece of cling film. Place alignment markers onto the backing card, e.g. use special fluorescent patches or radioactive ink.
8. Expose the filters to preflashed X-ray film and place in a cassette containing an intensifying screen. Leave at –70°C overnight or longer.

9. Develop the film and align it to the filters. Mark the plate alignment marks from the filters onto the film and match up the original agar plates to identify any positive plaques using the black marks from the needle (Section 17.3.9, step 4).

17.3.11 Rescreening

Usually it is impossible to isolate a pure clone after one round of screening so the positives identified after the primary screen are replated and screened again.

1. Using the wrong end of a sterile large tip or Pasteur pipette, core the agar around the area of the positive plaque and place it in 1 mL SM buffer containing a drop of chloroform.
2. Leave phage to diffuse for 1–2 h at RT.
3. Titrate the phage.
4. Replate the phage so that there are approximately 200 plaques or less on a 90 mm Petri dish. Repeat the screening procedure.
5. Identify a well isolated plaque or if necessary, screen again until this is achieved.
6. Place a single plaque into 200 μL SM buffer containing a drop of chloroform. Keep at 4°C.

Having identified a putative positive, it will be necessary to isolate DNA from it to check that the insert is responsible for the signal. One can then proceed to further analyse the clone.

NOTES

1. If necessary, allow the DNA to stand for several hours at 4°C after dilution to ensure its full dispersal. High molecular weight DNA should only be pipetted with a pipette tip which has had the end cut off to avoid shearing.
2. Occasionally partial digestion can be inhibited by factors still present in the DNA preparation, particularly when mild procedures are used to isolate the DNA. In this case, extract the DNA once with an equal volume of phenol: chloroform (1:1 v/v) and dialyse extensively against TE pH 7.6.
3. For small quantities of DNA, e.g. 10–20 μg, the optimum conditions for partial digestion are set up using less important DNA isolated in a similar manner. The sample is then digested under these conditions and an aliquot (0.5 μg) is analysed on a 0.5% gel. The reaction is stopped by freezing so that it is possible to add extra enzyme if necessary. The fragments can be treated with alkaline phosphatase to prevent multiple insertion.
4. If preferred the DNA can be: (i) digested with the restriction enzyme; (ii) extracted with an equal volume of phenol:chloroform and precipitated with ethanol; (iii) recovered and resuspended in 10 mM Tris-HCl pH 7.5 (0.5 μg/mL) and $MgCl_2$ added to a concentration of 10 mM. Incubate at 42°C for 1 h to promote the annealing of the *cos* ends; (iv) check that the arms have annealed by running a sample on a gel at low voltage. Use heated cleaved arms as a marker; (v) load the sample onto a salt gradient.

5. If one is using arms which have not been purified, allow a little extra in the reactions to compensate for the molarity being affected by the presence of the stuffer fragment.
6. If the plaques touch there is a chance that the bacteria will be simultaneously infected with more than one phage which may lead to genetic recombination.
7. Top agarose is favoured when taking nitrocellulose lifts as it is less likely to adhere to the filters.
8. If you are not sure at what stringency to wash the filters, it is best to err on the side of caution as the filters can always be washed again at a higher stringency after exposure to X-ray film. Low stringent washes are carried out at high salt concentration and/or low temperature. Progressively increasing the temperature and reducing the salt concentration will increase the stringency to a maximum of $0.1\times SSC$ and 1% SDS at 68°C for DNA probes.

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CHAPTER EIGHTEEN

Constructing and screening cosmid libraries

Kostas D. Mathiopoulos

18.1 INTRODUCTION

A genomic library is a nearly complete set of genomic DNA fragments that can be screened with molecular probes. For most organisms, no prior detailed information about the genome is required for library construction. The ability to clone and propagate DNA fragments of various sizes has been of paramount assistance in the elucidation of genome organization, gene structure and regulation, as well as in the detection and analysis of sequence polymorphisms.

Traditionally, libraries of complex genomes have been constructed in λ phage or cosmid vectors using *Escherichia coli* as a host. Plasmid vectors are somewhat less useful due to the difficulty of obtaining consistently large inserts. Instead, packaging of recombinant DNA molecules into λ phage heads provides an excellent selection for inserts of maximum length of 20–25 kb, whereas cosmid vectors accept an average insert size of about 40 kb. Recently, the use of yeast artificial chromosomes (YACs) has extended the range of the possible insert sizes up to 1000 kb or more (Burke *et al.*, 1987). For a review on genomic libraries in λ and cosmid vectors, refer to Frischauf (1991).

Essential in screening a library is to begin with a high enough number of clones in order to increase the chances of selecting the desired clone. If the genome size of an organism is known (Chapter 16), and assuming DNA inserts of nearly identical size and complete randomness of sequence representation, the number of clones needed to be screened (N) can be approximated by the formula (Clarke and Carbon, 1976):

$$N = \frac{\ln(1-p)}{\ln(1-x/y)}$$

where x is the insert size, y is the size of the haploid genome, and p is the probability of a given sequence being present in the library. For example, for a genome size like that of *Drosophila* of about 1.7×10^8 bp, an average insert size of 17 kb (in a λ phage library) and $p = 0.99$ we obtain $N = 4.6 \times 10^4$. Alternatively, ~20 000 colonies would need to be screened if a cosmid library of a ~40 kb insert size were used. Note that this number represents approximately ~4.5 genome equivalents.

This chapter describes the construction and screening of cosmid libraries, since it provides a combination of larger insert size with relative ease of construction and

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minimum laboratory requirements, while the previous chapter contains all the necessary information for the construction of libraries in bacteriophage λ. However, when choosing between a λ and a cosmid library one should have in mind the relative advantages and disadvantages. The major advantage of using a cosmid vector is due to the greater length of DNA fragments that can be cloned (~ 45 kb). The benefits are three-fold. First, a modest reduction in the number of recombinants required for a representative library. Second, reduction in the number of steps required to walk along a genome. Third, the possibility of including in a single DNA fragment certain, particularly long, eukaryotic genes.

On the other hand, the disadvantages of cosmid systems are mainly on two fronts. First, cosmid systems are still not as efficient as λ vectors in the recovery of recombinant DNA molecules. This is particularly relevant when supplies of donor DNA are limiting. It should never be forgotten that considerably higher molecular weight DNA is required as starting material for efficient cloning in cosmids than in λ. Second, screening a cosmid library by colony hybridization is less efficient than screening a λ library by plaque hybridization. This reflects, in part, the reduction in plasmid copy number associated with an increase in plasmid size. Modern cosmid systems, however, are less affected by insert size and also less subject to deletion, a problem to which cosmid recombinants are notoriously prone.

Vector systems are continually evolving, but novel systems take time to be developed, assessed and approved. The choice remains the researcher's, who should consider both specific needs and material availability.

18.2 MATERIALS

18.2.1 Media

1. LB (Luria-Bertani) broth: Dissolve 10 g Bacto-tryptone (Difco), 5 g Yeast extract (Difco) and 5 g NaCl in 600 mL deionized water. Adjust to pH 7.2 with NaOH. Bring to 1 L with water and sterilize by autoclaving. Keep at RT. If solution gets cloudy (indicating contamination), discard.
2. LB plates: Add 15 g Bacto-agar (Difco) per L of LB broth and sterilize by autoclaving. Pour appropriate sized plates. If ampicillin needs to be added (50 μg/mL), cool agar to about 55°C before adding it.
3. LB top agar: Add 7 g agarose per L of LB broth and sterilize by autoclaving.

18.2.2 Host strains

1. *NM554*: Alternatively, XL1-Blue MR or AG1 strains can be used for cosmid cloning. The important point is that a strain be *recA*– (and therefore help stabilize cosmid clones) and deficient in most of the *E. coli* endogenous restriction systems (thus protecting methylated DNA).

18.2.3 Reagents and buffers

1. Antibody-AP conjugate: Dilute anti-digoxigenin-AP conjugate to 150 mU/mL (1:5000) in buffer 2 (see below).
2. Blocking reagent stock solution: 10% powdered skimmed milk in buffer 1 (see below). It is prepared by dissolving 10 g powdered milk in 100 mL buffer 1 by

stirring and gentle heating. Avoid boiling as it may cause the milk to coagulate. Do not sterilize. You can freeze for months or keep at 4°C for a week or two. Being milk, it will eventually turn sour.

3. Buffer 1: 100 mM maleic acid, 150 mM NaCl. Adjust to pH 7.5 with NaOH.
4. Buffer 2: 1% powdered skimmed milk in Buffer 1.
5. Buffer 3: 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$. The pH at 9.5 is important but do not adjust the pH to 9.5 after you have made up the solution, otherwise $Mg(OH)_2$ will precipitate resulting in high, spotted background. To prepare 100 mL buffer 3, add 10 mL of 1 M Tris pH 9.5, 10 mL of 1 M NaCl and 5 mL of 1 M $MgCl_2$ to 75 mL deionized water.
6. 10× CIP dephosphorylation buffer: 10 mM $ZnCl_2$, 10 mM $MgCl_2$, 100 mM Tris-HCl, pH 8.3.
7. Colour substrate solution: 45 μL nitroblue tetrazolium salt (75 mg/mL in 70% dimethylformamide) and 35 μL X-phosphate (50 mg/mL 5-bromo-4-chloro-3-indolyl phosphate, toluidinium salt in 100% dimethylformamide) in 10 mL buffer 3. Make fresh each time.
8. Denaturation solution: 0.5 M NaOH, 1.5 M NaCl.
9. Homogenization solution: 10 mM Tris-HCl pH 7.5, 60 mM NaCl, 10 mM EDTA, 0.15 mM spermine, 0.15 mM spermidine.
10. Hybridization buffer: 5×SSC, 0.02% SDS, 1% Blocking reagent from Blocking reagent stock solution.
11. Neutralization solution: 0.5 M Tris-HCl pH 8.0, 1.5 M NaCl.
12. 10× nick-translation buffer: 500 mM Tris-HCl pH 7.8, 50 mM $MgCl_2$, 0.5 mg/mL bovine serum albumin, 100 mM dithiothreitol.
13. Probe stripping solution: 0.2 N NaOH, 0.1% SDS.
14. 20×SSC: 3 M sodium chloride, 0.3 M sodium citrate.
15. 10% SDS: dissolve 10 g of sodium dodecyl sulfate in 100 mL deionized water. Use the hood for weighing SDS, or wear a face mask.
16. SM buffer: To 800 mL deionized water add 5.8 g NaCl, 2 g $MgSO_4.7H_2O$, 50 mL 1 M Tris-HCl pH 7.5 and 5 mL 2% gelatin and stir. Make up to 1 L with deionized water, sterilize and store in 50 mL aliquots at 4°C.
17. TE: 10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0.
18. Sodium acetate: For a buffered 3 M solution, dissolve 408.2 g sodium acetate.$3H_2O$ in about 800 mL deionized water, adjust to pH 5.2 with glacial acetic acid, and bring to 1 L. Sterilize by autoclaving.

18.2.4 Enzymes

1. Restriction enzymes: *Bam*HI, *Xba*I, *Sau*3A, provided with their 10× reaction buffers.
2. Modification enzymes: T_4 ligase, calf intestine phosphatase (CIP), DNase I, DNA polymerase I (holoenzyme), provided with their reaction buffers.
3. Other enzymes: proteinase K.

18.2.5 Solvents

1. Chloroform:isoamyl alcohol: 24:1 (v/v).
2. Equilibrated phenol: phenol must be equilibrated to pH>7.8 because DNA will partition into the organic phase at acidic pH. Extract several times with TE pH

8.0 before adding to chloroform:isoamyl alcohol. Store at 4°C for 1 month or keep frozen aliquots indefinitely.
3. Equilibrated phenol:chloroform:isoamyl alcohol: 25:24:1 (v/v/v).

18.2.6 Other materials

1. Nitrocellulose or nylon filters. Use precut filters, their diameter depending on the size of the plates to be used. Approximately 35% fewer colonies can be plated on 87 mm filters, but the small filters are somewhat easier to handle than the 137 mm ones. The decision is purely a matter of individual taste. The filters should be detergent-free!
2. Whatman 3MM filter paper.

18.3 METHODS

Cosmids are cloning vectors, usually 5–8 kb in length, that bear *cos* sites of bacteriophage λ. They determine the ability of source DNA in the size range 37–52 kb to be packaged into λ heads, leading to the formation of infectious particles. The elements must be present at both ends of the DNA. Recombinant molecules are thus about 40 kb inserts flanked at both ends by cosmid sequences. Several cloning vectors are available (e.g. pWE15, Lorist B, Lawrist4, SuperCos 1, pJB8, pcos1EMBL) that offer a combination of different selective markers and different cloning sites. For simplicity reasons, the SuperCos 1 vector (Stratagene) will be used as a reference in the remainder of this chapter (Wahl *et al.*, 1987). It is a 7.6 kb long vector using the ampicillin gene as selective marker. Its cloning site is *Bam*HI which is flanked by *Not*I sites, allowing the excision of most inserts as a single restriction fragment. Moreover, it contains bacteriophage promoter sequences flanking the *Bam*HI cloning site, which permits the rapid synthesis of 'walking' probes. It is designed for cloning using very small amounts of genomic DNA. Finally, it contains multiple *cos* sites and a unique restriction site between them that allows simple and efficient cloning using nonsize-selected DNA (Figure 18.1). In terms of library construction, little has changed from the Ish-Horowicz and Burke (1981) procedure. Briefly, the major steps are:

1. The DNA of a cosmid is cleaved with appropriate restriction enzyme(s) and, if needed, treated with phosphatase to suppress self-ligation.
2. High molecular weight genomic DNA is partially digested with a 'frequent-cutter' restriction enzyme that creates termini compatible with the restriction enzyme used to cleave the cosmid vector. Fragments of average length 35–45 kb are selected from this population by gel electrophoresis or velocity gradient centrifugation (in the case of SuperCos 1 this step is unnecessary).
3. The vector DNA and the fragmented eukaryotic DNA are ligated to form long concatameric molecules that are subsequently packaged *in vitro* into bacteriophage λ heads. The recombinant phage are used to infect *E. coli*. Cosmid-containing bacteria are then selected by virtue of their ability to grow in the presence of the appropriate antibiotic. The cosmid library can then be maintained as a

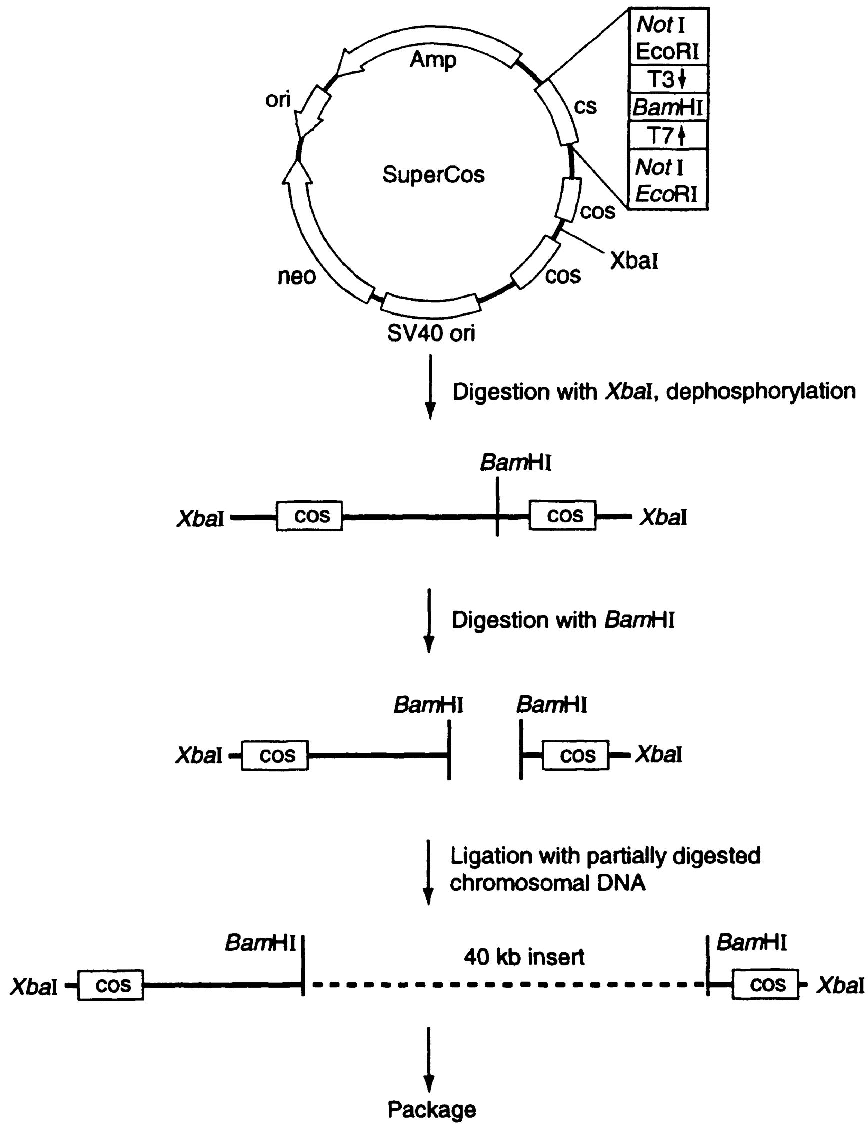


Figure 18.1 The principles behind cloning and packaging using a cosmid vector. The vector (SuperCos) is first linearized by restriction with a unique enzyme (*Xba*I) and then dephosphorylated with calf intestine phosphatase. The vector is then restricted with the unique enzyme at its cloning site and ligated with partially restricted genomic DNA. Several combinations are possible, but only those that result in 37–52 kb products can be packaged. Abbreviations: *cos*, *cos* site; Amp, ampicillin resistance gene; *neo*, neomycin resistance gene; cs, cloning site; *ori*, *E. coli* origin of replication; SV40 ori, replication origin in Simian Virus 40.

population of bacteria (as individual colonies on filter or, usually, pooled and stored in a freezer).

18.3.1 Isolation of high molecular weight chromosomal DNA

Isolate the DNA as described in Chapter 14. Determine its concentration by measuring the A_{260}. Check the quality of the DNA (i.e., approximate size and RNA contamination) by electrophoresis on a 0.5% agarose gel (Note 1).

18.3.2 Library construction

(a) Preparation of genomic DNA

1. Partial digestion of genomic DNA. Mix 100 μg of genomic DNA with 5.0 units of *Sau*3A in 1× *Sau*3A digestion buffer in a total volume of 120 μL. Transfer to a 37°C water bath and incubate. At 7, 10 and 15 min intervals remove 40 μL aliquots and transfer to precooled microfuge tubes containing 2 μL 0.5 M EDTA to stop the reaction. Pool the DNA, phenol/chloroform extract and ethanol precipitate. Resuspend in 95 μL water by gentle mixing. Check 5 μL on a 0.5% agarose gel. Make sure that the partial digestion has yielded the fragment sizes desired (Note 2).
2. Dephosphorylation of partially digested genomic DNA. To the digested genomic DNA add 10 μL 10× calf intestine phosphatase (CIP) buffer and 2 μL CIP (1 U/μL) and incubate at 37°C for 30 min. Add an additional 2 μL CIP and incubate for 30 min more. Inactivate the enzyme by heating at 70°C for 15 min. Extract DNA with phenol/chloroform and ethanol precipitate as usual. Resuspend DNA in 50 μL TE.
3. Determine the concentration of genomic DNA either by measuring the A_{260} or by electrophoresis on a 0.5% agarose gel. Genomic DNA is then ready for ligation with vector. It can be stored at 4°C. With the use of the SuperCos 1 vector, no additional size fractionation is necessary since only ligation products of between 37 and 52 kb can be packaged.

(b) Preparation of vector

When preparing a cosmid library it is important to avoid the inclusion of more than one vector molecule per recombinant. In vectors with appropriate restriction sites the polymerization of vector molecules can be prevented by cutting with different combination of restriction enzymes to create two different arms which are prepared separately (Ish-Horowicz and Burke original protocol). Alternatively, if a vector with two *cos* sites and a unique restriction site between them is used (like SuperCos 1), it is possible to restrict between the *cos* sites, treat with alkaline phosphatase, then cut with the enzyme at the cloning site and use the vector without physical separation of arms.

1. Digestion of vector DNA with *Xba*I. Digest 20 μg of vector with 200 U of *Xba*I enzyme in a total volume of 200 μL in standard buffer conditions for 1 h at 37°C. Extract once with phenol/chloroform and once with chloroform. Add 1/10 vol 3 M sodium acetate pH 5.5 and 2.5 vol of ethanol to precipitate the DNA. Wash pellet with 70% ethanol, air-dry and resuspend in 95 μL water (final concentration ~200 ng/μL). Check 5 μL for complete digestion on a 0.8% agarose gel. You should be able to see a single 7.6 kb long band corresponding to the linearized cosmid.
2. Dephosphorylation of digested vector. Add 10 μL 10× CIP buffer and 2 μL CIP and incubate at 37°C for 30 min. Add an additional 2 μL and incubate for 30 min more. Inactivate the enzyme by heating at 70°C for 15 min. Extract DNA with phenol/chloroform and ethanol precipitate as usual. Resuspend DNA in 50 μL TE.
3. Digestion of dephosphorylated vector with *Bam*HI. Digest dephosphorylated vector with 100 U of *Bam*HI in standard conditions in a total volume of 200 μL for

1 h at 37°C. Proceed with a phenol/chloroform extraction and an ethanol precipitation. Resuspend the pellet in 20 μL TE. If there have been no losses, this would correspond to a final concentration of ~1 μg/μL. Run 1 μL on a gel to check concentration and also digestion. You should see two bands (1.1 kb and 6.5 kb).

4. The vector is thus ready for ligation with genomic DNA. It can be stored at –20°C indefinitely.

(c) Ligation and packaging

1. Ligate 2.5 μg genomic DNA with 1.0 μg of SuperCos 1 vector (Stratagene) into its *Bam*HI site. Set up the reaction as follows:
   2.5 μg Prepared genomic DNA
   1.0 μg Prepared SuperCos 1 vector
   2.0 μL 10× Ligation buffer
   H_2O to 20 μL
   Mix by gentle tapping on the tube and centrifuge for a few seconds to bring the droplets to the bottom of the tube. Remove 1 μL that will be used later to test ligation. Add 1 μL (400 Weiss U) of T_4 ligase, mix by pipetting gently and incubate overnight at 14°C. Remove 1 μL to test the ligation (Note 3).
2. Packaging of cosmid DNA. Use commercial packaging extracts. GigapackII λ XL packaging extract (Stratagene), for example, yields 10^6 recombinant clones per μg of source DNA. Other commercial extracts give similar yields. If the cost of the commercial packaging extracts is prohibitive, they can still be prepared in the laboratory, following the protocols described by Sambrook *et al.* (1989). Follow the manufacturer's instructions for packaging the DNA using 1/10 of the ligation reaction. Do not attempt to package more as the titre will drop. Raise the volume of the packaged phage to 500 μL with SM buffer. Add 40 μL chloroform and mix by gentle inversion. Centrifuge briefly at low speed in a microfuge to pellet cell debris. Packaged material can be stored for up to 6 months at 4°C without loss of infectivity.
3. Estimate the unamplified titre of the cosmid library. Prepare fresh host cells (NM554). Start a 50 mL overnight culture in LB broth with 0.2% maltose and 10 mM $MgSO_4$ from a single colony. Spin down cells for 10 min at 4000 rpm at 4°C. Resuspend in 12.5 mL 10 mM $MgSO_4$ and store at 4°C until needed (do not store more than 2 days). Just before use, dilute cells to $A_{600} = 0.5$ in 10 mM $MgSO_4$. Make a 1:10 and a 1:50 dilution of the packaged DNA in SM buffer. Mix 25 μL of each dilution with 25 μL of host cells in a microfuge tube and let sit at RT for 30 min. Add 200 μL LB to each sample and incubate for 1 h at 37°C shaking the tube gently once every 15 min to allow expression of the ampicillin resistance gene. Plate cells on LB plates containing 50 μg/mL ampicillin. The titre should be between 1×10^5 and 1×10^6 transformants per μg of chromosomal DNA. If the titre is good the remainder of each ligation may be packaged and titred.
4. Amplifying the library. Incubate an amount of the packaging mixture corresponding to approximately 3×10^5 packaged cosmids with 600 μL of fresh host cells. Incubate for 20 min at 37°C to allow attachment of the bacteriophage particles to the bacteria. Add 3 mL LB and incubate for a further 45 min at 37°C. Apply 500–600 μL of the infected cell culture to the centre of each of six LB plates containing 50 μg/mL ampicillin. About 5×10^4 colonies/plate should be obtained after an overnight incubation at 37°C. Scrape the colonies off the plates with LB

broth, mix with ampicillin (50 μg/mL final) and glycerol (15% final). Plate a small aliquot with several serial dilutions to estimate the titre. Amplified libraries can be stored at –80°C for several years without a substantial drop of the titre. Amplified libraries tend to be less representative, requiring the plating of larger numbers of colonies to find a particular clone.

18.3.3 Library screening

(a) Immobilization of DNA on membrane filters and hybridization analysis

1. Place a membrane filter on the plates to be used for the library plating. Plate the library directly on the plate with the membrane. Plate enough bacteria to obtain a high enough number but not confluent colonies. Make enough plates to screen 4–5 genome equivalents.
2. Prepare duplicate plates. When the colonies are visible remove the membrane filter with the bacterial cells from the plate and place it onto a piece of Whatman 3MM paper with a pair of flat-ended forceps, the bacterial colonies facing up. Prewet a new filter by placing it on a LB plate. Remove it from the plate and carefully overlay it onto the first filter with the bacterial cells. Place a second sheet of 3MM paper and a glass plate over the filters and apply pressure to evenly transfer the cells from one filter to the other. Remove glass plate and top 3MM paper and with a syringe needle make three or four asymmetrical holes that will be later used for correct alignment of the filters. Peel replica filter and return on the agar plate, cells facing up. Incubate for 6–8 h at 37°C or until the colonies are visible. A second replica should be made out of the same original. To make the exact same holes with the needle, the two filters should be turned over. Up to four replicas can be easily produced from the same original, each time increasing the pressure on the glass plate during the transfer. The original filter should be layered face-down (!) on a fresh plate for 5 min and peeled off. The plate is incubated at RT overnight and then stored at 4°C until the screening is finished.
3. Immobilize DNA on the filters. After the cells are grown, remove the filter from the plate and lyse the cells by placing it for 3 min on Whatman 3MM paper soaked in denaturing solution. Transfer the filter onto a second sheet for 3 min more. Then neutralize the filter by placing it for 3 min on 3MM paper soaked in neutralization solution. Transfer onto second 3MM paper for 3 more min. Briefly wash off excess salt from the filters by rinsing them in 2×SSC (Note 4). Air-dry the filters and immobilize the DNA either by baking at 80°C for 2 h or by crosslinking the DNA using a UV crosslinker. After that, gently rub off cellular debris from the filters using gloves in a solution of 2×SSC and 0.1% SDS. If needed, filters are best stored wrapped up with aluminium foil at 4°C.

(b) Probe labelling

Various combinations of labelling approaches and marker molecules can be used to label a probe. If the probe to be labelled is in a plasmid vector, however, the first step should be the purification of the insert fragment from the plasmid vector since vector sequences share homology with vectors used in the library construction and thus result in a very high background that renders the identification of the positive clones

impossible (Note 5). Several labelling methods can be found elsewhere in this book (Chapters 17 and 20). Here I describe a nonradioactive nick-translation approach that results in probes of high quality.

1. Nick-translation. The nick-translation reaction (Rigby *et al.*, 1977) utilizes the ability of DNA polymerase I to add nucleotides sequentially to the 3′-end of a nick within a DNA duplex, while removing nucleotides from the adjacent 5′-terminus. Nicks are introduced in a random manner by the addition of pancreatic deoxyribonuclease I (DNase I), so that the net effect is to produce a uniformly labelled population of molecules.
2. Prepare the following: nucleotide mix (0.5 mM dATP, dGTP and dCTP each), 10× nick-translation buffer, enzyme mix (1 μg/mL DNase I and 10 U/mL DNA polymerase I).
3. Mix the following in a microfuge tube on ice:

DNA solution	100 ng–1 μg
Nucleotide mix	4 μL
10× Nick-translation buffer	5 μL
1 mM DIG–11-dUTP (Note 6)	10 μL
Water to bring the volume to	48 μL
Enzyme mix	2 μL

4. Mix gently by pipetting up and down. Incubate at 15°C for 1–2 h. Terminate the reaction by adding 2 μL 0.5 M EDTA. Remove unincorporated nucleotides by ethanol precipitation.

(c) Hybridization

1. Hybridization. Wet filters in hybridization solution. Transfer filters in hybridization bags or tubes or any appropriate containers and prehybridize them at 65°C for at least 1 h. Denature probe by boiling in a water-bath for 5–10 min. Cool immediately on ice for 5 min. Mix probe in hybridization solution. Hybridize at the same temperature from 6 h to overnight.
2. Posthybridization washes. Wash filters twice in 2×SSC, 0.1% SDS at RT for 5 min and twice in 0.1×SSC, 0.1% SDS at hybridization temperature (65°C) for 30 min with constant shaking.

(d) Signal detection

1. Colour development. Equilibrate membranes in buffer 1 for 1 min. Block membranes by gently agitating in buffer 2 for 30 min. Incubate membranes in antibody-AP conjugate solution for 30 min. Wash antibody solution twice with buffer 1 for 15 min each. Equilibrate membranes in buffer 3 for 2 min. Add colour substrate to the membranes and incubate in the dark until colour has developed (Note 7).
2. Chemiluminescent detection. If higher sensitivity is required, Lumigen PPD [Boehringer, 10 mg/mL (23.5 mM) Lumigen PPD diluted 1:100 in buffer 3] can be used instead of the colour substrate for chemiluminescent detection. In this case, dilute the antibody-AP conjugate solution 1:10 000 (75 mU/mL) instead of 1:5000. After equilibration in buffer 3 for 2 min, place the membrane between two sheets of acetate, lift the top sheet and add diluted Lumigen over the surface distributing it evenly. Lower the top acetate sheet and wipe off the excess liquid. Then

place the membrane with the acetate sheets in a sealed plastic bag and incubate for 15 min at 37°C in the dark. Expose for about 60 min to standard X-ray film; this should be sufficient to detect a single-copy clone (Note 8).

3. Align the filters with the autoradiogram and label the latter at the positions corresponding to the holes. Place the plate containing the library on the autoradiogram (if signal detection is by chemiluminescence) or the colour-developed membrane (if signal detection is by colour development) and align them carefully. Using sterilized toothpicks transfer the bacteria from the areas corresponding to the signal into a tube with 0.5 mL of LB broth and replate (with suitable dilutions). Use a separate series of dilutions for each positive signal. Repeat the steps 1 to 5 until a single colony can be picked over a positive signal.

(e) Stripping membranes for reprobing

If the probe is to be stripped off the filter membranes so that they can be reused in a subsequent screening, the filters should not be allowed to dry as it will not be possible to remove the probe. Only nylon membranes can be stripped since nitrocellulose is dissolved by the reagents used to remove colour.

1. Remove the colour by incubating the membranes in heated dimethylformamide (50–60°C) with several changes until the desired decolorization has been achieved.
2. Rinse membranes in water and incubate twice for 10 min in probe-stripping solution at RT.
3. Rinse the membranes thoroughly in 2×SSC and either store (they can now be allowed to dry) or continue with prehybridization.

NOTES

1. Preparation of very good quality genomic DNA is probably the most important step for a representative cosmid library. Check the size of DNA on a 0.5% agarose gel or by pulse-field gel electrophoresis. Do not attempt to construct a library unless the average size of the DNA exceeds 150–200 kb. One needs to be careful and very gentle when handling DNA since it is easily sheared.
2. In order to determine the ideal conditions for obtaining the desired insert size range (35–45 kb), a series of test partial digests are necessary. Set up a sample digest of 10 μg of genomic DNA in a 100 μL reaction using 1× *Sau*3A buffer. Add 0.5 U of *Sau*3A and remove 15 μL aliquots at various time points of the digestion (0, 5, 10, 20, 30 and 45 min time points). Check aliquots by electrophoresis on a 0.5% agarose gel to choose the optimal conditions. Scale up the reaction according to the results obtained.
3. To test the ligation, remove 1 μL before addition of the ligase and 1 μL after the reaction is complete and run on an agarose gel. If the ligation has been successful, some of the genomic DNA should have been converted to high molecular weight concatamers and a smear or a diffuse band should appear that should have migrated slower than the starting material. A band corresponding to linear cosmid monomers should still be apparent.

4. When processing many filters at once it is somewhat difficult to keep times. Only the length of the denaturation step is really critical.
5. In order to purify an insert from a plasmid clone, the insert should be first excised by digesting the clone with the appropriate restriction enzyme. This is followed by electrophoresis on a 1% agarose gel. The desired fragment can be isolated following any standard protocol.
6. For nonradioactive labelling, instead of DIG–11-dUTP, biotin-14-dUTP or biotin-16-dUTP are commercially available and work as well.
7. Do not shake the container while colour is developing. A positive signal should become visible after an incubation of 1–2 h, depending on the size of the colony.
8. For the briefest exposure to X-ray film, the AP chemiluminescent reaction must be at steady-state. Once steady-state is reached (15 min at 37°C or 7–8 h at RT) an approximate time of 15 min is required to detect a single copy colony. If the membrane is exposed before steady state is reached, a 60 min exposure may be required. Times of incubation and exposure to film should be determined empirically.

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CHAPTER NINETEEN

Constructing and screening cDNA libraries

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19.1 INTRODUCTION

The in-depth study of eukaryotic gene structure and expression has been made possible by the ability to clone transcribed DNA. Since RNA *per se* cannot be cloned (i.e. stably introduced and replicated in a bacterial cell) and RNA–DNA hybrids can only be cloned with very low efficiency, the best choice for the isolation of mRNA is its conversion into double-stranded cDNA. Thus, a good strategy for the isolation of a particular eukaryotic gene is the screening of a cDNA library representing the mRNA population of the appropriate tissue or cell type using an available nucleic acid as a probe. Alternatively, the clone of interest can be identified by virtue of the expression of a specific protein by the target mRNA. In the first case, the hybridization probe could be an existing cloned gene from the same or another species, an oligonucleotide or a pool of oligonucleotides (Suggs *et al.*, 1981), or a PCR product (Lee *et al.*, 1988). In the second approach expressed proteins can be detected by specific antibodies (Young and Davis, 1983b) or by functional assays that determine their biological activity (Yang *et al.*, 1986).

The choice of vector for cDNA cloning depends on the method which will be used for screening, on the ease with which the resulting clones can be grown and manipulated, and on the number of recombinants necessary to ensure the presence of the desired sequence. In general, for cDNA libraries the bacteriophage λgt10 (Young and Davis, 1983a) has been the preferred vector for screening by hybridization, while phage λgt11 (Young and Davis, 1983a; 1983b) is suitable for screening by binding of an expressed polypeptide to antibodies. However, several plasmid vectors are also available for either type of screening. Recently, a new generation of vectors has been designed (λZAP), combining the properties of λ and plasmid vectors (Short *et al.*, 1988). A considerable improvement has been the possibility to create unidirectional cDNA libraries (Okayama and Berg, 1982a, 1982b). Unidirectional libraries are constructed in such a way that all of the cDNA inserts are positioned with their 5′ ends adjacent to a strong promoter for eukaryotic expression. These libraries are extremely useful when detection of cloned genes is to be accomplished by antibody screening or through functional assays, although screening with nucleic acid probes is still possible.

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The faithful representation of the target mRNA population is essential in any library construction. Clearly, the representation of a cDNA clone of a particular mRNA species depends on the abundance of that species among the cellular mRNA. To isolate cDNA clones of rare mRNAs (less than 10 copies per cell) it is necessary to construct large cDNA libraries representative of complex poly(A)$^+$ RNA populations and, whenever possible, to start with tissue material already potentially enriched in the mRNA of interest. It has been estimated that about 2×10^5 recombinants must be screened (using nucleic acid probes) to detect a low-abundance mRNA from mammalian cells (Sambrook *et al.*, 1989). Given the fact that this number is estimated on the basis of a $\sim 3.3 \times 10^9$ bp genome, one can estimate the number of recombinants needed to be screened from an insect library depending on the size of its genome.

Independent of the use of a phage or a plasmid vector, the construction of a cDNA library starts by isolating mRNA and reverse transcribing it into cDNA using the enzyme reverse transcriptase. Following that, the concerted action of several *E. coli* enzymes (RNase H, DNA polymerase I and DNA ligase) convert the RNA–DNA hybrids into double-stranded cDNA molecules. Subsequent treatment with T_4 DNA polymerase ensures that polynucleotide linkers can be blunt-end ligated to the cDNA. These linkers make the cDNA compatible with the ends present on the cloning vector. After ligation, the actual library is established either by packaging the phage or transforming *E. coli* with the recombinant plasmids.

19.2 MATERIALS

19.2.1 Media

1. LB (Luria-Bertani) broth: Dissolve 10 g Bacto-tryptone (Difco), 5 g Yeast extract (Difco) and 5 g NaCl in 600 mL deionized or double-distilled water. Adjust to pH 7.2 with NaOH. Add water to 1 L and sterilize by autoclaving. Keep at RT. If the solution gets cloudy (indicating bacterial contamination), discard.
2. LB plates: Add 15 g Bacto-agar (Difco) per L of LB broth and sterilize by autoclaving. Pour appropriate size plates. If an antibiotic needs to be added (e.g. ampicillin 50 μg/mL), cool agar to about 55°C before adding it.
3. LB top agar: Add 7 g agarose per L of LB broth and sterilize by autoclaving.

19.2.2 Host strains

1. XL1-Blue.

19.2.3 Reagents and buffers

1. 25× agarase buffer: 750 mM BisTris, 250 mM EDTA.
2. Antibody-alkaline phosphatase (AP) conjugate: Dilute anti-digoxigenin-AP conjugate to 150 mU/mL (1:5000) in buffer 2 (Section 18.2.3).
3. Colour substrate solution: 45 μL nitroblue tetrazolium salt (75 mg/mL in 70% dimethylformamide) and 35 μL X-phosphate (50 mg/mL 5-bromo-4-chloro-3-indolyl phosphate, toluidinium salt in 100% dimethylformamide) in 10 mL buffer 3 (Section 18.2.3). Make fresh each time.

4. Denaturation solution: 0.5 M NaOH, 1.5 M NaCl.
5. First strand 5× reaction buffer: 250 mM Tris-HCl pH 8.3, 400 mM KCl, 15 mM $MgCl_2$. Store at 4°C.
6. Hybridization buffer: 5×SSC, 0.02% SDS, 0.1% N-lauroylsarcosine, 1% Blocking reagent from Blocking reagent stock solution.
7. 10× methylation buffer: 0.5 M Tris-HCl pH 7.5, 10 mM EDTA, 50 mM dithiothreitol.
8. Neutralization solution: 0.5 M Tris-HCl pH 8.0, 1.5 M NaCl.
9. Second strand 5× reaction buffer: 100 mM Tris-HCl pH 7.0, 20 mM $MgCl_2$, 450 mM KCl, 750 mM β-NAD, 50 mM $(NH_4)_2SO_4$.
10. 20×SSC: 3 M sodium chloride, 0.3 M sodium citrate.
11. SDS, 10%: dissolve 10 g of sodium dodecyl sulfate in 100 mL deionized water. Weigh SDS under the hood or wear a protective mask.
12. SM buffer: to 800 mL deionized water add 5.8 g NaCl, 2 g $MgSO_4 \cdot 7H_2O$, 50 mL 1 M Tris-HCl pH 7.5 and 5 mL 2% gelatin and stir. Make up to 1 L with deionized water, sterilize by autoclaving and store in 50 mL aliquots at 4°C.
13. TBST buffer: 10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05% Tween-20.
14. TE: 10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0.
15. 3 M sodium acetate: sodium acetate has a molecular weight of 136.08 (trihydrate). For a buffered 3 M solution, dissolve 408.2 g sodium acetate$\cdot 3H_2O$ in about 800 mL deionized water, adjust to pH 5.2 with glacial acetic acid, and bring to 1 L. Sterilize by autoclaving.

19.2.4 Enzymes

1. Restriction enzymes: *Eco*RI, provided with its 10× reaction buffers.
2. Modification enzymes: agarase, T_4 DNA ligase, T_4 DNA polymerase, *E. coli* DNA ligase, *E. coli* RNase H, DNA polymerase I (holoenzyme), *Eco*RI methylase, RNase inhibitor, M-MuLV reverse transcriptase, provided with their reaction buffers.

19.2.5 Solvents

1. Chloroform:isoamyl alcohol: 24:1 (v/v).
2. Equilibrated phenol: phenol must be equilibrated to pH>7.8 because DNA will partition into the organic phase at low pH (<6.0). Extract several times with TE pH 8 before adding to chloroform:isoamyl alcohol. Store at 4°C for 1 month or keep frozen aliquots indefinitely.
3. Equilibrated phenol:chloroform:isoamyl alcohol: 25:24:1 (v/v/v).

19.3 METHODS

Many approaches have been used to generate cDNA libraries that have tried to preserve as much of the original sequence as possible, to improve cloning efficiency, and to facilitate screening and subsequent analysis (for a review see Kimmel and Berger, 1987).

19.3.1 RNA preparation

The quality and quantity of cDNA synthesized by any method is critically dependent on the integrity of the mRNA used as the template. Template poly(A)$^+$ RNA should be prepared by any of the standard procedures available (Chapter 15). It does no harm to reiterate that extremely stable RNases are ubiquitous in the laboratory environment (including fingers of the researcher and, to various extents, in all cells) and therefore the precautions mentioned in Chapter 15 should be re-emphasized here as well. The RNA intactness and its contamination with DNA can be judged by denaturing gel electrophoresis.

19.3.2 cDNA synthesis

The classical method of cDNA synthesis took advantage of the 3′ hairpins generated by AMV reverse transcriptase during first strand synthesis; they were used to prime the second strand synthesis catalysed by Klenow DNA polymerase and reverse transcriptase (Efstratiadis *et al.*, 1976). After completion of the second strand, S1 nuclease was used to cleave the hairpin loop. This digestion was difficult to control and was the major cause of low cloning efficiencies and the loss of a significant amount of sequence information, especially towards the 5′ end of the mRNA.

A first improvement in cDNA synthesis was made by avoiding the use of S1 nuclease (Okayama and Berg, 1982a,b; Gubler and Hoffman, 1983). The first strand was synthesized in the presence of 4 mM sodium pyrophosphate which suppressed the formation of hairpins. Second strand synthesis was then carried out using RNase H to create nicks and gaps in the hybridized mRNA strand, providing 3′-OH priming sites for DNA synthesis and repair by DNA polymerase I. In this case, the second strand reaction can be carried out immediately following first strand synthesis by simple addition of the reagents to the original reaction mixture. After a brief incubation with T_4 DNA polymerase to remove any remaining 3′ protruding ends, the blunt-ended, double-stranded cDNA is ready for fractionation and addition of linkers or the adaptors. Unfortunately, RNase H activity that resides on some reverse transcriptases presented several drawbacks as, for example, was the degradation of RNA in the RNA–cDNA hybrids. Recently, a ribonuclease-free reverse transcriptase from Moloney murine leukaemia virus (M-MuLV) has become available with superior efficiency with respect to size and mass yields of the cDNA generated.

(a) First strand synthesis

1. Mix the following on ice:

Oligo $dT_{12–18}$ (500 μg/mL)	
or random hexamers (0.15 μg/mL)	1 μL
mRNA	2 μg
RNase inhibitor	25 U
Water, to a total volume of	10 μL

2. Heat the mixture to 70°C for 10 min to eliminate secondary structure of mRNA and chill on ice.
3. Add:

5× Reaction buffer	4 μL
0.1 M Dithiothreitol	2 μL
10 mM dNTPs	1 μL

[α-^{32}P]dCTP (>400 Ci/mM)	1 μCi
RNase H-free reverse transcriptase (Gibco-BRL), 200 U/μg of mRNA	

The final volume should be 20 μL. Mix gently and incubate at 37°C for 1 h, then transfer the reaction tube to ice.

4. Withdraw 1 μL of the first strand products, dilute 10-fold and use for the estimation of first strand yields. Keep the rest of the first strand reaction on ice and continue with second strand synthesis within 1 h (Note 1).

(b) Second strand synthesis

The second strand reaction is performed directly following the first strand synthesis without the need for extractions, precipitations or change of the reaction tube.

1. To the remaining 19 μL of first-strand reaction add the following (on ice):

5× Second strand buffer	32 μL
10 mM dNTPs	3 μL
0.1 M Dithiothreitol	6 μL
E. coli DNA ligase	15 U
E. coli DNA polymerase I	40 U
E. coli RNase H	1.4 U
Water to a final volume of	160 μL

2. Incubate the reaction at 16°C for 2 h, then transfer to ice.

(c) Creating blunt ends on the double stranded DNA

1. To the second strand reaction mix add 10 U T_4 DNA polymerase. Mix and incubate at 37°C for 10 min.
2. Terminate reaction by placing at RT and adding EDTA to a final concentration of 20 mM.
3. Perform one phenol/chloroform and one chloroform extraction. Add 20 μg carrier tRNA and precipitate with ethanol.
4. Resuspend in 16 μL distilled water and remove 1 μL for gel analysis and estimation of yield or recovery.

19.3.3 Preparation of cDNA for cloning

The cDNA needs to be made compatible with the corresponding vector ends. Either λgt10 or λgt11 require *Eco*RI ends, therefore phosphorylated linkers should be ligated to the cDNAs. Since the linkers, upon ligation, will form concatamers, the *Eco*RI overhangs have to be created by digestion with *Eco*RI restriction endonuclease. Sites internal to the cDNA should be first protected from digestion by methylation with *Eco*RI methylase. After digestion with *Eco*RI, the cDNAs are size-fractionated in order to remove free linkers and to select full-size cDNA molecules for cloning.

(a) Eco*RI methylation*

1. Mix the following on ice:

Double-stranded cDNA	15 μL (~1 μg)
10× Methylation buffer	2 μL

100 mM *S*-adenosyl methionine (SAM)	2 μL
*Eco*RI methylase	1 μL (~20 U)
Water to final volume of	20 μL

2. Incubate at 37°C for 15 min, then inactivate methylase by heating at 70°C for 15 min.
3. Phenol extract and ethanol precipitate as usual. Resuspend pellet in 5 μL water.

(b) Addition of linkers and EcoRI *restriction*

1. Mix the following on ice:

Methylated cDNA	5 μL (~1 μg)
Phosphorylated *Eco*RI linkers	2 μL (400 ng)
10× Ligase buffer	1 μL
T_4 DNA ligase	1 μL (~400 U)
Water to final volume	10 μL

2. Incubate at 4°C overnight. Heat-inactivate the reaction at 70°C for 15 min. Cool the tube on ice and add the following:

*Eco*RI 10× buffer	3 μL
*Eco*RI (20 U/μL)	2 μL
Water to final volume	30 μL

3. Mix and incubate at 37°C for 1 h. Ethanol precipitate as usual. Resuspend pellet in 10 μL water.

(c) Size-fractionation of cDNA

1. Load methylated cDNA on a 1% low melt agarose gel in TAE buffer and separate electrophoretically.
2. Visualize cDNA under long-wave UV light and using a sterile scalpel, cut the part of the gel that corresponds to molecular size cDNA above 0.8 kb.
3. Weigh the agarose fragment and add 25× agarase buffer to 0.04% of the weight of the piece of agarose. Heat at 65°C in a waterbath until the agarose is completely melted.
4. Gradually bring the temperature of the waterbath down to 45°C. Add 1 U of agarase per 100 mg agarose and incubate for 1 h (Note 2).
5. Cool on ice for 15 min. Centrifuge for 5 min and collect supernatant in a separate tube leaving behind undigested pieces of agarose. Agarose will often be completely digested and no pellet will be detected.
6. Continue by adding 1/10 vol 3 M sodium acetate and 2.5 vol ethanol and precipitate as usual.
7. Resuspend in water to obtain 50–100 ng/μL. The cDNA is now ready to ligate to dephosphorylated λ arms.

19.3.4 Preparation of vector for cloning

In choosing between plasmid and bacteriophage λ vectors, several factors must be considered. In general, λ vectors are preferred when large numbers of recombinants are needed. This is because high cloning efficiencies (10^7 pfu/μg arms) are consistently obtained *via in vitro* packaging reactions and subsequent infection of the bacterial host cells. In addition, it is possible to efficiently screen large numbers of recombinants

at high plaque densities with either nucleic acid or antibody probes. With plasmids, cloning efficiencies are usually 10–50 times lower, colonies must be screened at much lower densities, and screening is more difficult, especially with antibodies. Plasmids have been more attractive mainly because of the availability of a wider variety of cloning sites, possibilities for using tailed vector and vector-primer cloning strategies, and the ease of isolation and manipulation of the resulting clones. λZAP (Short *et al.*, 1988) is a new generation of vectors designed to provide an increased range of potential sites for cloning and expression of cDNA and a simplified method for the recovery and subsequent manipulation of cDNA. It carries a polycloning site downstream from an *E. coli lacZ* promoter, accepts up to 10 kb inserts that can be expressed in either infected bacteria or induced lysogens. λZAP fusion proteins may be expressed from high-copy number plasmids following excision of the DNA insert from the bacteriophage λ vector.

(a) Preparation of vector

In order to ligate the cDNAs to the λ arms, the vector needs to be digested with *Eco*RI as well. If λZAP or a plasmid vector is used instead, a different appropriate restriction enzyme can be chosen.

1. Mix the following on ice:

λ Vector of choice	5 μg
10× *Eco*RI restriction buffer	4 μL
*Eco*RI	10 U
Water to	40 μL

2. Mix and incubate at 37°C for 1 h. Heat at 70°C for 15 min.
3. Phenol/chloroform extract and ethanol precipitate.
4. Resuspend in 5 μL TE. Run 0.5 μL on a 0.8% agarose gel to check digestion.

19.3.5 Establishment of the library

(a) Ligation of cDNA to λ arms

Before packaging, the cDNAs should be first ligated to the vector of choice. Since the cDNAs are heterogeneous is size, different ligation ratios can yield somewhat different cloning efficiencies. The optimal ligation conditions can be established empirically. A rule of thumb is 50–100 ng of cDNA per 1 μg of λ arms in a 5 μL ligation reaction.

1. Mix the following on ice:

Sized cDNA	1 μL (~50 ng)
λ/*Eco*RI prepared arms	1 μL (1 μg)
10× Ligase buffer	0.5 μL
Water	2 μL

2. Incubate at 42°C for 15 min to anneal the cohesive λ ends (concatamer formation is necessary for optimal packaging).
3. Place the tube on ice and add 0.5 μL T_4 DNA ligase (200 U), giving a final volume of 5 μL. Mix and incubate at 14°C overnight.

(b) In vitro *packaging*

1. Use 1 μL from the 5 μL ligation per packaging reaction, following the instructions of the packaging mixes (see also Chapters 17 and 18).

2. To test the quality of this packaged ligation product plate 1 μL of the final 500 μL packaged reaction with 200 μL of A_{600} = 0.5 XL1-Blue host cells (Chapters 17 and 18).
3. Absorb the phage to the bacteria for 15 min at 37°C, to permit phage to attach to the cells, before adding top agar and IPTG/X-gal. Add 2–3 mL of melted top agar (48°C) plus IPTG/X-gal, plate immediately onto appropriate agar plates (Note 3).
4. Plaques are visible after incubation for 6–8 h at 37°C. Background plaques are blue and should be fewer than $1x10^5$ pfu/μg of arms, while positive plaques will be white and should be 10–100 fold above background.
5. If the test titre gives the expected results, package the rest of the sample ligation and titre.

(c) Amplification

1. Prepare XL1-Blue host cells as described in Chapter 17 and 18. You will need 600 μL of A_{600} = 0.5 cells per 150 mm agar plate.
2. Mix aliquots of the packaged λ clones or amplified suspension containing approximately 50 000 recombinant bacteriophage with 600 μL of host cells adjusted to an A_{600} of 0.5. If screening $1x10^6$ plaques, you will need 20 aliquots of 50 000 plaques each (Note 4).
3. Incubate tubes containing phage and host cells 15 min at 37°C.
4. Mix 6.5 mL of melted 48°C top agar with each aliquot of infected bacteria (make sure top agar is cooled to approximately 48°C before adding to aliquot) and pour evenly onto the appropriate 150 mm plate of bottom agar. Do not use IPTG or X-gal.
5. Incubate plates at 37°C for 5–8 h. Do not allow the plaques to get too large.
6. Pipette 10 ml of SM buffer onto each plate. Incubate the plates at 4°C overnight with gentle shaking or rocking.
7. The next day recover the bacteriophage suspension from each plate and pool into sterile polypropylene or glass container. Rinse the plates with an additional 2 mL of SM and add to the pool. Add chloroform to 5% and invert to mix. Incubate 15 min at RT.
8. Remove cell debris by centrifugation for 5 min at 4000 g.
9. Recover the supernatant and transfer to sterile polypropylene or glass bottle. Add chloroform to 0.3% and store in aliquots at 4°C.
10. Check the titre of the amplified library using host cells and serial dilutions of the library. It will usually be in the range of approximately 10^{10}–10^{12} pfu/mL.

19.3.6 Screening

(a) DNA screening – immobilization of the cDNA library onto membrane filters

1. In a typical DNA screening, host cells are infected with a recombinant library and plated. Plate with host cells and 6.5 mL of top agar per plate on the appropriate 150 mm plates (approximately 2-day-old plates) to approximately 50 000 plaques/plate (use 20 plates to screen 10^6 plaques). Use approximately 600 μL of A_{600} = 0.5 host cells XL1-Blue per large plate titre (Note 5).
2. Grow at 37°C. If plating late in the day, it is possible to incubate the plates overnight, otherwise incubate plates 6–8 h (XL1-Blue cells will usually require overnight growth).

3. Refrigerate plates for 2 h at 4°C to chill. This helps prevent top agar from sticking to the nitrocellulose filter.
4. Transfer plaques for 2 min onto labelled (with pencil) nitrocellulose (or nylon) filters. Make 3–4 asymmetrical holes by pricking the filter with a needle through the agar for orientation. The marking by the needle can be improved by first dipping it into black indian ink. If a duplicate filter is to be used, the second transfer should last for approximately 4 min. Following the marks that the needle left on the agar, make the same holes on the second filter. Use forceps and wear gloves during handling the filters.
5. Denature nitrocellulose filter after careful lifting by submerging it in denaturation solution for 2 min. Neutralize for 5 min by submerging in neutralization solution. Rinse filters for 1 min in 0.2 M Tris-HCl, pH 7.5, 2×SSC. All solutions should be at RT.
6. Air-dry filters on Whatman 3MM filter paper and immobilize the DNA on them either by baking for 2 h at 80°C or by crosslinking the DNA by a UV irradiation.
7. Probe labelling and subsequent screening of the cDNA library can be performed as described in Chapters 17 and 18.

(b) Immunoscreening

λ expression vectors have become increasingly popular for the construction of cDNA libraries, especially because of their ability to express high levels of the desired products. Cloning into the *lacZ* gene can result in the expression of foreign DNA as part of a β-galactosidase fusion protein which can be detected with antibodies. Thus, a rabbit or mouse primary antibody can be used as a probe to detect recombinants containing DNA sequences of interest.

In a typical immunoscreening, host cells are infected with a recombinant library and plated. After 3.5 h of growth at 42°C, the plates are overlaid with nitrocellulose (or nylon) filters saturated with IPTG, an inducer of *lacZ* gene expression, and incubated for another 3.5 h at 37°C. During this time, the plaques are transferred to the filters along with β-galactosidase fusion proteins released from lytically infected cells. The filters are removed from the plates and blocked by incubation in a solution containing excess protein to saturate nonspecific protein binding sites on the filter. The filters are then incubated with a primary antibody directed against the antigen of interest, washed, and incubated with the appropriate (anti-rabbit or anti-mouse) secondary antibody–alkaline phosphatase conjugate. After a final wash, colour development substrates are added. Usually within a few minutes, positive plaques turn dark purple in colour as a result of alkaline phosphatase activity. Positive plaques are then located and removed from the original plates, and the recombinant plaques are purified in a secondary screening. In more detail, the protocol is as follows:

1. Infect fresh host cells with the recombinant library and plate on 150 mm plates as described above. Incubate at 42°C for 3.5 h.
2. Move plates to a 37°C incubator. Carefully (avoiding air bubbles) overlay each plate with a dry nitrocellulose filter disk which has been saturated previously in 10 mM IPTG in water. Incubate for 3.5 h at 37°C. If a duplicate is required, a second filter may be overlaid after the first has been removed. Incubate for an additional 3 h at 37°C. Incubation of the nitrocellulose filter may also be performed overnight at 4°C or, if the plaques are not yet large enough, at RT.

3. Chill the plates at 4°C for 20 min, mark the position of the filter on the plate with a needle, and carefully remove the filters.
4. Place filters in TBST buffer and rinse briefly to remove any remnants of agar. Do not allow the filters to dry out during any of the subsequent steps.
5. To saturate nonspecific protein binding sites, incubate in TBST + 20% calf serum or other suitable protein blocking agent (e.g. 1% BSA, gelatin or casein) for 15–30 min. Use 15 mL per 132 mm filter.
6. Incubate the filters for 30 min in TBST + primary antibody (Notes 6 and 7). Use 15 mL per 132 mm filter. Use serum antibody or ascites fluid at a 1:200–1:1000 dilution. The positive control mouse anti-β-galactosidase antibody can be used at a 1:5000 dilution. The diluted primary antibody can generally be reused several times (Note 8).
7. Wash the filters 3 times, 5–10 min each, in 20 mL TBST buffer.
8. Transfer the filters to TBST containing the appropriate second antibody–alkaline phosphatase conjugate (a 1:7500 dilution is recommended). Allow 15 mL per 132 mm filter. Incubate at RT for 30 min.
9. Wash the filters 3 times, 5–10 min each, in 20 mL TBST buffer.
10. Blot the filters damp dry on filter paper and transfer to freshly prepared AP colour substrate solution. Positive clones will appear as purple plaques on the white filters. Development of colour will continue for at least 4 h. Filters can be developed overnight, but this may result in high background.
11. When the colour has developed to the desired intensity, stop the reaction by placing the filters in 20 mM Tris-HCl, pH 8.0, 5 mM EDTA. The filters can be stored in this solution or dried. The colour will fade slightly upon drying but it can be restored by moistening the filter with water.
12. To further purify a putative positive signal, remove an agar plug containing phage particles from the region of the plate corresponding to the signal on the filter. Incubate the agar plug with 1 mL of λ diluent for at least 60 min at RT with occasional vortexing. Replate the phage and repeat the screening procedure. This time it should be easier to identify many more well-isolated positive plaques for further analysis.

(c) Rescreening

A rescreen is necessary in almost all cases. Even with the best alignment of the autoradiogram and the plate that was used for screening it is difficult to pinpoint to a single plaque unless the density is very low. Thus, the area around the positive signal should be scratched off the plate and, after appropriate dilutions, it should be rescreened. Only 100–200 plaques/plate are necessary for rescreening.

NOTES

1. The newly synthesized cDNA strand will be labelled to facilitate tracing the product in the following reactions. At each step about 1% of the product should be set aside to check the size distribution of the cDNA in a denaturing gel.
2. When the percentage of agarose is higher than 1% the amount of agarase enzyme has to be adjusted proportionally. When using Tris-borate gel electrophoresis buffer, a two-fold amount of agarase should be used.

3. Use IPTG/X-gal only to determine the background numbers. Do not use IPTG/X-gal in the amplification or DNA screening protocol since the IPTG may induce expression of your cloned cDNA, which could be toxic to the host cell.
4. The amount of packaged material should be less than 100 μL.
5. If the plates are too wet while plating, the top agarose may detach itself while lifting the filter. Some smearing may also be observed. Remove moisture by leaving the tops off of the plates for 10–15 min before plating at 37°C. Do not let the plates get too dry or plaques may grow too small. Small plaques could also be a result of too high a concentration of plating cells.
6. The quality of the antibody probe is very important. High titre, high affinity antibodies produce better signals than low titre, low affinity antibodies. A purified IgG fraction will sometimes work better than whole antiserum and an affinity-purified antibody generally produces the best results. In general, antibodies that produce good signals on Western blots will produce good signals in the screening procedure at similar dilutions.
7. Most primary antibody preparations can be used in the 1:1000 dilution range. Unnecessarily high concentration of some antisera can accentuate backgrounds produced by anti-*E. coli* antibodies which may be present at low titres.
8. If little or no signal is obtained or if the background is too high in the screening procedure, the antiserum or ascites should be checked on Western or dot–blots before proceeding. A few antibodies, particularly some monoclonals, are conformation-dependent and do not recognize antigens immobilized on a surface. Others may tend to stick nonspecifically to surfaces such as nitrocellulose. These antibodies are not suitable for screening with this technique.

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CHAPTER TWENTY

Southern/northern blotting and hybridization techniques

Inga Sidén-Kiamos

20.1 INTRODUCTION

Southern blotting was named after E.M. Southern (Southern, 1975) who first developed the basic technique. The method allows the transfer of DNA fragments to a membrane filter after having been previously separated by agarose gel electrophoresis. The subsequent hybridization of the filter with a radioactively labelled nucleic acid leads to the detection of such gel-separated fragments that share similarities with the probe. The northern blot in turn (the name is an obvious pun) represents the transfer of RNA, separated by electrophoresis, to a membrane filter, which is again hybridized with a labelled probe. This way one can identify transcripts that correspond to a specific DNA segment.

Both methods have found a wide use in molecular biology. For example, the Southern blot is commonly used for the restriction mapping of cloned genes, the identification of homologous DNA sequences in other species, the mapping of mutations in genomes, the estimation of the number of copies of a given sequence etc. Similarly, the northern blot is used to identify transcription units and direction of transcription and, among other applications, it has enabled the detection of specific transcripts during different developmental stages.

The general steps are similar for both techniques. The first step is the electrophoretic separation of DNA or RNA by agarose gel electrophoresis. Usually DNA samples will previously have been digested with (a) suitable restriction enzyme(s). After the electrophoresis is completed the nucleic acid is transferred to a membrane filter by capillary blotting. The filter is then hybridized with a radioactively labelled probe. The conditions are chosen so that hybrids will be formed between fragments bound to the filter and homologous (or near-homologous) fragments of the probe. Free probe and nonspecifically bound probe is removed by washing. The filter is then ready for exposure to an X-ray film.

20.1.2 Agarose gel electrophoresis

The relative mobility of nucleic acid fragments in an agarose gel depends on the molecular size of the fragments. The distance they migrate from the well is inversely pro-

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portional to the $\log_{10}$ of their molecular size. Therefore the length of any DNA or RNA fragment can be determined provided that a set of DNAs or RNAs of known molecular weight have been run in parallel on the same gel. For DNA restriction digests this standard can, for example, consist of phage λ DNA that has been restricted with a suitable enzyme. Other parameters that will influence the mobility are the concentration of agarose, the conformation of the DNA fragments (linear, nicked circles or closed supercoiled circles) or the secondary structures formed by RNA molecules and the current applied. Agarose gels are usually run horizontally.

20.1.3 Blotting

The transfer of the nucleic acid from the gel to the membrane filter is usually performed by capillary blotting, although a faster method using vacuum has been developed. This technique needs special equipment and the manufacturer supplies the specific protocol. Therefore the technique is not dealt with here. The capillary transfer method is simple and cheap, its only drawback being the time required since it usually proceeds overnight. Briefly, the gel is placed on a support that also functions as a reservoir for transfer buffer; this support could be a stack of papers, a wick connected to a buffer reservoir or a sponge. A membrane filter is placed on the gel, a pack of dry absorbing papers are added and finally a weight is put on top of the setup. The capillary force will draw the buffer up to the dry papers together with the nucleic acid. The nucleic acid will be bound to the filter, under the condition that it is single-stranded and a buffer of high ionic strength is used. The latter is especially important when using nitrocellulose filters, while for nylon filters other buffer systems can be used according to the conditions recommended by the manufacturers. To obtain single-stranded DNA the gel is treated with NaOH before transfer, while for RNA such a step is in fact harmful as it will degrade the RNA.

The time for complete transfer depends on several factors, such as the thickness of the gel, the concentration of agarose and the size of the fragments being transferred. Fragments in the size of a few kilobases (kb) are transferred from a 1% gel within a few hours, while fragments larger than 10 kb take several hours. Small fragments (<500 bp) bind to nitrocellulose with very low affinity, requiring special buffers although they bind with somewhat higher efficiency to nylon filters. As a rule of thumb plasmid digests can be blotted for a few hours, while it is advisable to let genomic digests and RNA transfer overnight.

After the transfer has been completed it is important to fix the bound nucleic acid to the filter. This can easily be done by baking the dried filter in an oven at 80°C. Alternatively, nylon filters can be crosslinked by exposing to UV light. Either a special UV crosslinking box (available from Stratagene or Amersham) can be used for this, or a UV lamp that has been previously calibrated for this purpose. UV crosslinking, rather than baking, is recommended if the filters are to be reprobed many times.

20.1.4 Hybridization

The theory of hybridization is highly complex and not easily transferred to the practical situation of filter hybridization, where a number of factors are unknown. A brief description of parameters of importance are given below for guidance.

1. Temperature: The choice of temperature for hybridization must be such that stable hybrids are formed between homologous, but not heterologous, sequences. The T_m or melting temperature is the temperature where a double-stranded hybrid is denatured into its complementary single-strands (while heating in solution), or conversely where two complementary single-strands will form a double-stranded helix (when a solution of denatured nucleic acids is cooled). The T_m depends on the G+C content of the nucleic acids in the hybrid and the concentration of Na^+. The T_m is about 80–90°C in 0.18 M Na^+. The T_m also depends on the number of mismatches in the hybrid. Usually the temperature for hybridization and washing is set at 20°C below the T_m. In aqueous solution (and in the ionic strength described below) 65°C is therefore a good choice when looking for homologous pairing. If one wishes to allow hybrids with mismatches to form, one can decrease the hybridization and washing temperatures to 60 or 55°C. Below this temperature spurious hybridization will give a very high background.
2. Concentration of probe: The lower the probe concentration the slower is the hybridization rate. Usually the amount of labelled probe available is limited and it is therefore important to keep the volume of the hybridization solution at a minimum level. This is especially important if the complexity of the probe is very large (complexity being the total sum of unique sequences). However it is important to add enough solution so that the filter is covered and, if done in plastic bags, any bubbles can move around freely.
3. Ionic strength: For hybridization to occur the concentration of Na^+ must be high. This is due to the fact that in the absence of a positive charge the negative charge of the phosphate groups of the sugar-phosphate backbone repel each other. Under these conditions no annealing can take place. Increasing the Na^+ concentration will increase the formation of duplexes, allowing mismatched hybrids to be formed. Adjustment of the Na^+ concentration is especially valuable when using a heterologous probe, where one can decrease the stringency of the washing by increasing the ionic strength.
4. Complexity of the probe: The formation of hybrids will be slower when the complexity of the probe increases. This will only interfere in a substantial way if the probe is >100 kb.
5. Size of the probe: The molecular size of the labelled molecules need to be small in order for the probe to access the bound nucleic acid. This is achieved by the random priming method for making probes described here, but also when nick-translation is used to label DNA probes.
6. Formamide: By performing the hybridization in a solution of 50% formamide the T_m of the hybrids is decreased. The hybridization may therefore be carried out at a lower temperature. This is of practical importance for northern blots, where the RNA on the filter may be degraded at high temperatures. The disadvantages are the toxicity of formamide, which is a teratogen, and the decreased rate of annealing, probably by a factor of 2–3× in 50% formamide. Formamide is therefore not recommended for Southern blots.
7. Before performing the actual hybridization with the probe it is important to block nonspecific binding sites on the filter. This is done by prehybridizing the filter in a solution containing a blocking reagent. Usually the same solution is used for prehybridization and hybridization. Different blocking reagents can be used, for example bovine serum albumin (BSA), sodium dodecyl sulfate (SDS),

dry milk, salmon sperm DNA, tRNA, Denhardt's reagent. The choice of blocking reagent depends on the membrane, the probe and the abundance of the target on the membrane.

20.2 MATERIALS

20.2.1 Agarose gel electrophoresis (for DNA)

1. Agarose (Low EEO grade).
2. Ethidium bromide: 10 mg/mL. Store at 4°C in a dark bottle.
   Note! Ethidium bromide is a mutagen! All solutions and gels containing ethidium bromide should be handled wearing gloves and suitable protective clothing and discarded as separate waste according to your local regulations.
3. TBE running buffer (10×): 0.89 M Tris-HCl, 0.89 M boric acid, 0.02 M EDTA. Store at RT. The buffer should be checked occasionally as salt sometimes precipitates. This does not seem to interfere, in most cases, with the buffer capacity if one takes care not to transfer the precipitate to working solutions.
4. Gel loading buffer (6×) : 0.25% bromphenol blue, 0.25% xylene cyanol, 15% Ficoll (type 400) in H_2O. Store at RT.
5. Polaroid film: Type 57 or type 667.

20.2.2 Formaldehyde agarose gel (for RNA)

1. Agarose (Low EEO grade).
2. MOPS running buffer (10×): 0.2 M MOPS, 50 mM sodium acetate, 10 mM EDTA, pH adjusted to 7.0 with NaOH. Store at RT protected from light. The buffer will turn yellow if exposed to light, which does not seem to interfere with its function, unless it becomes dark brown.
3. Formaldehyde, check that pH > 4.0.
   Note! Formaldehyde is toxic by inhalation of the vapour and will penetrate skin. Work in a fume hood and wear gloves.
4. Formamide. Ultrapure formamide is available commercially, but a less expensive alternative is to deionize formamide of lower quality. Deionize by stirring with a mixed-bed ion-exchange resin, (e.g. Dowex XG8) for 1 h, then filter through filter paper. Store at –20°C.
   Note! Formamide is a teratogen and should be handled with care.
5. Bromphenol blue.

20.2.3 Southern/northern blotting

1. Denaturation solution: 1.5 M NaCl, 0.5 M NaOH.
2. Neutralization buffer: 1.5 M NaCl, 1 M Tris-HCl pH 8.0.
3. 20×SSC: 3 M NaCl, 0.3 M sodium citrate, adjust pH to 7.0 with few drops of a 10 N solution of NaOH.
   All solutions are stored at RT.
4. Whatman 3 MM paper.

5. Paper towels.
6. Membrane filter, nitrocellulose (available from many companies), or nylon filters, e.g. Hybond N (Amersham), Gene Screen (DuPont), Zeta probe (BioRad).

20.2.4 Random priming and G-25/G-50 spun column

1. TM: 250 mM Tris-HCl pH 8.0, 25 mM $MgCl_2$, 50 mM λ-mercaptoethanol.
2. DTM: 0.1 mM dGTP, 0.1 mM dTTP, 0.1 mM dATP in TM.
3. OL: 1 mM Tris-HCl pH 7.5, 1 mM EDTA pH 7.5, 90 A_{260} U/mL random hexamers. Random hexamers are available from a number of suppliers, e.g. Pharmacia.
4. LS: 25 μL 1 M Hepes pH 6.6, 25 μL DTM, 7 μL OL.
5. α-^{32}P-dCTP (specific activity about 600 Ci/mmol).
   Note! ^{32}P is a β emitting radioactive isotope. Consult your local regulations for working with radioactivity. Wear safety glasses, gloves and protective clothing. Protect yourself and the environment from radiation by working behind a shield. Clearly mark all items that are radioactive. Dispose of radioactive waste according to local regulations.
6. DNA Polymerase I-Klenow fragment (5 U/μL).
   All reagents are kept at –20°C.
7. Sephadex G-25 or G-50 (Fine or Medium, from Pharmacia) is resuspended in TE buffer (10 mM TrisHCl, 1 mM EDTA, pH 8.0). The resin should be 'swelled' either by autoclaving for 20 min or storing at RT overnight. Store at +4°C.
8. 1 mL hypodermic syringe.
9. Glasswool or fibrewool for aquarium pumps.

20.2.5 Hybridization (Church) system

1. Church phosphate buffer (1 L): 0.5 M Na_2HPO_4. Set to correct pH with 4 mL 85% H_3PO_4. Sterilize by autoclaving.
2. 20% SDS (sodium dodecyl sulfate).
3. 0.5 M EDTA pH 8.0.
   Store the above solutions at RT.
4. 10% BSA (bovine serum albumin), fraction V. Store at –20°C.
5. Salmon sperm DNA (optional), dissolve the DNA by stirring over low heat, shear the DNA by passing it with a syringe through a 17 G needle about 10 times. Store at 20°C. Boil just before use.
6. Church prehybridization/hybridization solution (50 mL), make fresh

Stock	Volume
20% SDS	17.5 mL
Church phosphate buffer	25 mL
10% BSA	5.0 mL
0.5 M EDTA	0.1 mL
Water up to	50 mL

7. Low stringency Church wash (high salt)

Stock	Volume
20% SDS	125 mL
Church phosphate buffer	25 mL
Water up to	500 mL

8. High stringency Church wash (low salt)

Stock	Volume
20% SDS	25 mL
Church phosphate buffer	25 mL
Water up to	500 mL

The Church wash solutions can be stored for weeks at RT.

20.2.6 Formamide system for northern hybridization

1. Formamide (Section 20.2.2)
2. 20×SSPE: 3 M NaCl, 0.2 M NaH_2PO_4, 20 mM EDTA, adjust pH to 7.4 with NaOH. Store at RT.
3. 50× Denhardt's reagent: (per L) 10 g Ficoll 400, 10 g polyvinylpyrrolidone, 10 g BSA fraction V. Slowly dissolve by stirring over low heat. Store at –20°C.
4. 20% SDS.

20.3 METHODS

20.3.1 Preparation of an agarose gel for DNA

1. Add the required amount of agarose to the correct volume of buffer, depending on the size of the gel box used (Notes and Figure 20.1). For most purposes a 1% agarose gel will be suitable. For very small fragments (<500 bp) increase the concentration of agarose (to maximum of 2%), and for very big fragments (>10 kb) decrease the concentration of agarose (to a minimum of 0.4%).
2. Boil the agarose for a few of minutes, until the agarose melts.

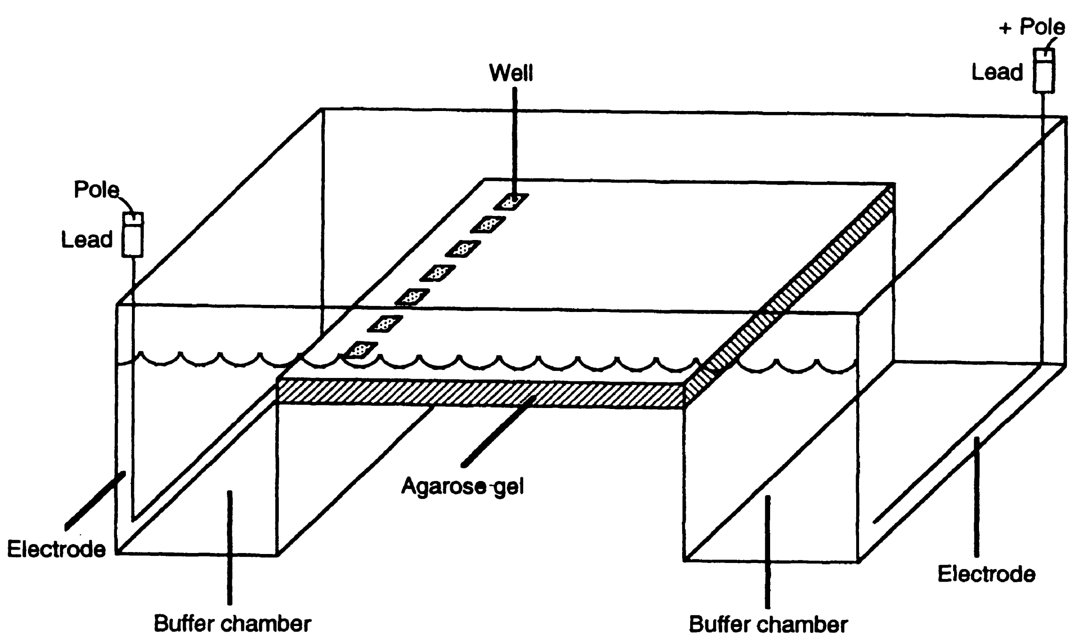


Figure 20.1 Diagram of the apparatus used for agarose gel electrophoresis.

3. Cool the solution to ~50°C and add ethidium bromide to a final concentration of 0.5 μg/mL. Alternatively, the gel can be stained in a water bath with ethidium bromide (0.5 μg/mL) after the end of electrophoresis.
4. Prepare the gel tray which should be clean and dry. Seal the ends carefully with tape. A comb should be inserted so that wells are formed. Pour the warm agarose solution into the tray. Allow to harden for about 30 min.
5. Remove the comb and the tape and put the gel in the electrophoresis tank.
6. Add sufficient electrophoresis buffer to slightly cover the gel. Connect the leads so that the samples will migrate towards the positive pole.
7. Mix the samples with the gel loading buffer and load. The amount of DNA to be loaded depends on the purpose of the experiment. 50–100 ng will be sufficient for a plasmid digest, while 2–10 μg should be loaded for a genomic Southern blot depending on the genome size of the species being analysed.
8. Run the gel at (typically) 50–100 V until the blue dye has migrated to about 75% of the length of the gel.
9. View the gel on a long wave UV transilluminator.
   Note! UV light can damage your eyes and skin. Wear suitable glasses and a face shield, and minimize exposure.

20.3.2 Formaldehyde gel electrophoresis of RNA

For general information on how to prepare and run horizontal agarose gels, see Section 3.1. For northern analysis the samples loaded are either total RNA or poly(A)$^+$ RNA. Their isolation is described in Chapter 15.

1. Make sure that the gel tank, comb and gel tray are clean and not contaminated with RNases. Prepare the tray.
2. For a gel of 100 mL (midigel): Melt 1 g of agarose in 72 mL of water. Cool to about 60°C, add 10 mL of 10× MOPS running buffer and 18 mL of 37% formaldehyde. The final volume can be increased or decreased depending on the size of the gel required. Pour the gel solution into the tray and allow the gel to set.
   Note! Formaldehyde vapours are toxic, prepare the gel in a fume hood.
3. Transfer the gel to the gel tank, add 1× MOPS running buffer and remove the comb carefully. Gels containing formaldehyde are brittle and the wells may easily break.
4. Prepare the samples. Mix 5.5 μL of RNA in H_2O (0.5 to 30 μg), 2 μL 10 × MOPS running buffer, 3.5 μL formaldehyde, 10 μL formamide, 0.4% bromphenol blue. Heat for 15 min at 65°C.
5. Load the samples. Connect the leads so that the RNA will migrate to the positive electrode. Run the gel at 3–5 V/cm for no more than 5 h (the formaldehyde will be depleted during longer runs). If the gel is run for more than 2 hours it is recommended that the running buffer is re-circulated between the buffer chambers by using a peristaltic pump. The electrophoresis is complete when the tracking dye has reached at least 50% of the gel.
6. At this stage the gel can be stained with ethidium bromide to visualize the RNA pattern on the gel (Section 20.4.3 for a discussion of ethidium bromide staining).

Alternatively ethidium bromide (1 μg/sample) can be added to the samples just before loading the gel.

20.3.3 Photography

Photographs of gels are usually taken using transmitted UV light (preferably a source with medium wave length 312_{nm}). The most sensitive film is Polaroid Type 57 or 667 (ASA 3000). With an efficient UV source (>2500 μW/cm^2) and a good lens, an exposure of less than a second is sufficient to obtain images of bands containing as little as 10 ng of DNA. To obtain better images of bands, there are two filters between the UV source and the lens: (i) transilluminator UV filter and (ii) Kodak 22A Wratten filter.

20.3.4 Southern blotting

1. After the end of electrophoresis, trim away any unused areas of the gel with a razor blade and cut off one corner for orientation.
2. Take a photograph of the gel placing a ruler along the side. This makes it easy to later compare the autoradiogram with the picture of the gel.
3. Soak the gel in denaturation solution for about 60 min at RT.
4. Soak the gel in neutralization buffer for about 60 min at RT.
5. Cut 5 Whatman 3MM papers to the same size as the gel.
6. Prepare the membrane (nylon or nitrocellulose) as follows (always handle the membrane with forceps or wearing gloves):
   a. cut a piece of membrane to the same size as the gel;
   b. float the membrane on the surface of a dish filled with 2×SSC until it wets completely from beneath.
7. Set up the transfer (Figure 20.2).
   a. wet the Whatman 3MM papers in 10×SSC (20×SSC for nitrocellulose filters) and stack 2–3 of them on a piece of foil or in a tray;
   b. place the gel on top of the 3MM papers. Take care to remove all bubbles;
   c. carefully layer the membrane filter onto the gel avoiding any misplacements; avoid any movements after its contact with the gel; cut the filter in the same corner as the gel.
   d. put the two remaining 2 Whatman papers on top of the membrane;
   e. put a stack of paper towels (6–8 cm) on top of the 3MM papers;
   f. put a weight on top of the paper towels, for example a glass plate with a bottle on top.
8. Allow the transfer to proceed 8–24 h (at least 16 h for genomic DNA transfer). When the transfer is complete gently remove the paper towels and the two 3MM papers. Remove the gel (which now has become very thin) and the filter as a unit. Mark the position of the wells on the filter with a pencil or ball point pen. The gel can now be peeled off the filter and discarded.
9. Rinse the filter briefly in 6×SSC to remove excess salt and any pieces of adhering agarose.
10. Dry the filter for at least 30 min at RT.

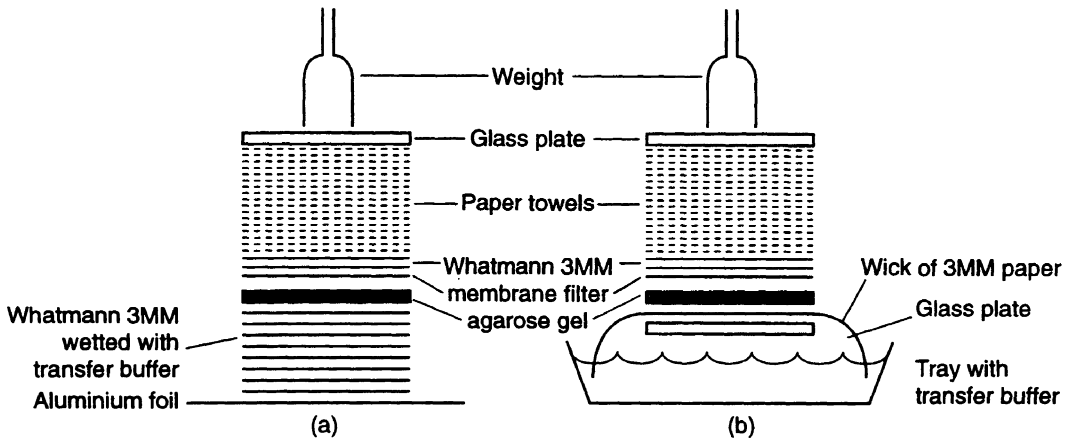


Figure 20.2 Diagram of the setup for Southern blotting. (a) The simplified protocol as described in the text, and (b) using a buffer reservoir system to improve the transfer.

11. Sandwich the filter between two sheets of dry 3MM paper. Fix the DNA to the filter, either by baking for 2 h at 80°C or by crosslinking with UV light.
12. The filter can be stored at RT until use.

20.3.5 Northern blotting

1. After the end of electrophoresis the gel is rinsed several times in H_2O, to remove formaldehyde which will otherwise interfere with transfer. Soak the gel in 20×SSC for 45 min. Proceed to setting up the transfer.
2. The transfer is set up as for Southern transfer, using 20×SSC as transfer buffer. Transfer is allowed to proceed for about 16–20 h (overnight).
3. After the transfer is complete, the filter is very briefly rinsed in 6×SSC, dried at RT and fixed as for DNA filters (baking at 80°C or crosslinking with UV).

20.3.6 Labelling DNA by random priming (probe preparation)

The random priming procedure is presented here for labelling with radioactive precursors (Feinberg and Vogelstein, 1983). Alternative labelling procedures can be found elsewhere in the book (Chapters 16 and 17).

1. Dilute 50–100 ng DNA in a total volume of 10.6 μL H_2O.
2. Boil the DNA for 5 min and immediately transfer to ice.
3. Add 11.4 μL LS, 2 ml α-^{32}P-dCTP and 1 μL Klenow (5 U/μL).
4. Incubate for 4 h to overnight at RT.
5. Separate the unincorporated labelled nucleotides by passing the sample through a G-25 or G-50 Sephadex column.
   Note! ^{32}P is a β-emitting radioactive isotope. Consult your local regulations for working with radioactivity. Wear safety glasses, gloves and protective clothing. Protect yourself and the environment from radiation by working behind a shield. Mark all radioactive items. Dispose of radioactive waste according to local regulations.

20.3.7 Sephadex G-25/G-50 column

1. Block from inside the outlet of a 1 mL syringe with glasswool or fibrewool.
2. Fill the syringe to the top with Sephadex G-25 or G-50 equilibrated in TE.
3. Place the syringe in a 15 mL centrifuge tube and centrifuge at 2000 rpm in a bench top centrifuge for 5 min at RT.
4. Place a microfuge tube (without the cap) under the syringe, put both into the 15 mL tube. Add the DNA sample to the top of the column. A suitable volume for the DNA sample is 100 μL. Avoid using more than 1/10 of the packed gel volume!
5. Again centrifuge at 2000 rpm for 5 min at RT. Collect the solution from the microfuge tube in the bottom.
6. To check for incorporation of a radioactive probe: count the DNA from the random priming reaction with the Geiger counter at a suitable distance, after the spun column count the probe (in an microfuge tube) at the same distance. You should expect to have at least 30% incorporation.

20.3.8 Hybridization of Southern blots

The basic protocol of Church and Gilbert (1984) is presented here.

1. Place the baked filter in the container to be used for hybridization. The filter can be wet in Church phosphate buffer (diluted 25-fold) to be more easy to handle, the buffer should be removed before prehybridization solution is added.
2. Prehybridization: Add Church prehybridization/hybridization solution (without probe). If plastic bags are used air-bubbles must be removed and the bag must be carefully sealed. Prehybridize for at least 10 min to overnight in a water bath or an oven at the desired temperature. Typically 65°C is used for homologous probes.
3. Discard the prehybridization solution and add a suitable volume of fresh Church solution. The volume of the hybridization solution should be sufficient to keep the filter wet, typically a volume of between 2 and 20 mL is used (the exact volume depends on the size of the filter, on the number of filters being hybridized simultaneously and on the type of container used).
4. Denature the labelled probe by boiling for 5 min. Immediately add it to the hybridization solution in the bag or bottle. Allow hybridization to proceed at the desired temperature for 16–24 h.
5. Discard the hybridization solution as radioactive waste and wash the filter under low or high stringency conditions. Do not allow the filter to dry out at any point before the washing is complete.
   The washing procedure is typically as follows:
   a. 1–2 short washes at RT to remove free probe. Discard these washes as radioactive waste;
   b. 2 × 30 min at the desired temperature. Monitor the washing with the Geiger counter, stop the washing when a part of the filter where no DNA is present is close to background.
6. Dry the filter slightly (do not dry completely if the filter is to be stripped and reprobed) at RT, wrap it in Saran wrap and expose on an X-ray film (Kodak XAR-5 or similar) using intensifying screens. Expose at –80°C for the desired time (a few hours to several days). With some experience and by checking with a Geiger counter one can get a fair estimation of the exposure time necessary.

7. Filters can be stripped and reprobed many times (nylon membranes are preferred when filters are to be reprobed as they are more robust than nitrocellulose). If filters are to be stripped and reprobed they must not be completely dried after hybridization. For stripping, pour boiling 0.5% SDS over the filter and allow to cool. The efficiency of stripping can be checked by exposing the filter.

20.3.9 Hybridization of northern blots

Northern blots can be hybridized using the Church system as described for above (Section 20.3.8) or in a formamide containing solution at a lower temperature (42°C) described here. The main steps and precautions are the same as for hybridization of Southern blots.

1. Wet the filter in 5×SSPE.
2. Prehybridize the filter in prehybridization solution: 50% formamide, 5×SSPE, 2× Denhardt's reagent, 0.1% SDS for a few hours to overnight at 42°C.
3. Remove the prehybridization solution and add fresh hybridization solution of the same composition as above.
4. Boil the probe for 5 min. Chill in ice or immediately add to the hybridization solution.
5. Hybridize at 42°C overnight.
6. Wash the filter briefly in 1×SSC, 0.1% SDS at RT to remove unbound probe. Repeat once.
7. Wash the filter in 0.2×SSC, 0.1% SDS at 65°C for 20 min. Repeat 2–3 times. If the probe is not homologous to the target RNA decrease the stringency by washing at a lower temperature or by using a washing buffer of higher ionic strength. If a radioactive probe is used, monitor with the Geiger counter, as for Southern blots.
8. Dry the filter (if the filter is to be stripped and reprobed leave it slightly moist), wrap it in Saran wrap and expose.

NOTES

Agarose gel electrophoresis

Horizontal gel boxes for agarose gel electrophoresis usually come in three different sizes, 'mini' (size of gel 5 × 7.5 cm, number of wells 5–10, loading capacity 5–15 μL), 'midi' (size of gel 12 × 14 cm, number of wells 10–20, loading capacity 10–30 μL) and 'maxi' (size of gel 20 × 25 cm, number of wells 15–30, loading capacity 25–50 μL). Note that the figures are approximate, and that each experimenter may wish to design his/her own gel boxes, depending on specific requirements. The loading capacity depends on the thickness of the gel (i.e. total gel volume), on how deeply the comb is inserted and on the size of the teeth of the comb. The gel boxes are made of Plexiglass and can either be ordered from your local workshop or purchased from various commercial sources.

The size of the agarose gel depends on the number of samples, the resolution required and the DNA analysed. A minigel set-up is sufficient for identifying frag-

ments of interest from a subclone, while a genomic Southern where one wants greater resolution should be prepared using a maxi gel apparatus.

Working with a high concentration of agarose and small fragments (up to a few kb) one can run the gel at fairly high voltage (100 V for a minigel) with no lack of resolution. However, with larger fragments and a lower concentration of agarose care should be taken to run the gel at low voltage for extended periods of time. For example a gel of 0.4% agarose can be run to resolve DNA fragments of up to 40 Kb, but should be run at 20 V for 20 h. If run at high voltage for many hours, recycle the buffer between the gel reservoirs at least once.

Southern blotting

The blotting procedure described in Section 20.3.4. is suitable for most experiments. However when large fragments (>15 kb) are blotted, a couple of added precautions may prove helpful. First of all it is important to break up large fragments while in the gel. This can be done by acid hydrolysis (soak the gel in 0.2 M HCl for 10–30 min, or until the bromphenol blue band has turned yellow, plus 10 min). The disadvantage of acid hydrolysis is that it may break the fragments into small pieces that do not stick to the membrane and it should therefore not be used when the fragments of interest are smaller than 15 kb. Second, the transfer can be improved by connecting a reservoir of buffer to the 3 MM paper under the gel (Figure 20.2b). This is simply done by putting the gel-filter stack on top of a glass plate, bridging a tray with buffer. A wick of 3MM paper connects the stack to the buffer. In this setup it is important to make sure that there is no contact between the paper towels and the wick, or otherwise the transfer of the DNA will be impaired. This is achieved by covering the free 3MM paper on the glass plate with strips of Saran wrap.

A note of caution. The nucleic acid in the gel will stick to the membrane filter after a very short time of contact (seconds!). Therefore once the filter is placed on top of the gel do not attempt to change its position.

When the blotting is performed using 10× or 20×SSC it is important to briefly rinse the filter after the transfer to remove excess salt. Otherwise the filter will become yellow and brittle after baking, which may also happen if the filter is baked for extended periods of time.

Northern blotting

A formaldehyde gel that is to be blotted is usually not stained with ethidium bromide, as the stain is thought to decrease the transfer of the RNA by a factor of 2–3. However, this may only be of practical importance if the mRNA studied is of low abundance. Stain the gel in 1× MOPS running buffer and one drop of ethidium bromide for 30 min with very gentle shaking. Destain in 1× MOPS running buffer for another 30 min. Photograph and proceed with transfer. If you work with a rare mRNA, run an extra lane that is cut away from the rest of the gel and stain this part of the gel.

DNA markers are not reliable as size markers for northern blots as DNA fragments will migrate more slowly than RNA of equal size. RNA markers are available commercially, e.g. from Boehringer Mannheim.

Random priming

The same protocol can be used for labelling with two radioactive nucleotides, or one nucleotide of a specific activity of 3000 Ci/mmole, if one wishes to have probes of higher specific activity. The same protocol can also be used for labelling with nonradioactively labelled nucleotides (biotin, digoxigenin). The DTM buffer should obviously be changed accordingly.

Hybridization

Hybridization can be performed in plastic bags sealed with a special heat sealer, in plastic or glass containers in a water bath or oven (in this case take care that your hybridization solution will not evaporate by covering the surface of the solution with Saran wrap), or in glass bottles in a 'rotisserie' oven. The last method is recommended as it gives an even distribution of fluid, less hybridization solution is required, many filters can be hybridized in one bottle (using the special nets provided by the manufacturer between the filters) and it also reduces the risk of contamination when using a radioactive probe.

Common problems

One of the most common problems in Southern/northern blots is a high background. Although experience says that the real cause of the background is never found, it could be due to one or more of the following reasons:

1. The kind of membrane used (try another batch, a different brand or 'classical' nitrocellulose).
2. The membrane was moved after it was placed on the gel.
3. The filter was not washed after completion of transfer.
4. The filter (or part of it) dried out during the hybridization and washing procedure.
5. Unincorporated nucleotides were not removed from the probe.
6. The filter was not prehybridized for long enough time.
7. The hybridization solution was not correct (check recipe and pH).
8. The filter was not washed for enough time or at high enough temperature.
9. 'Sticky' probe: some probes repeatedly give a high background, using a subclone or an adjacent fragment may alleviate the problem. One can also try to preadsorb the probe in the hybridization solution to a piece of unused filter overnight. However in this case the probe will have re-annealed and the probe in the hybridization solution will need to be denatured again before being added to the proper filter (by heating at 95°C for 10 min: boiling is not recommended since BSA will precipitate, although this may not change its properties).
10. Blocking reagents not optimal. For genomic Southern 100–200 μg/mL of single-stranded salmon sperm DNA may give an improvement.

The other common problem encountered in nucleic acid hybridizations (although not as often as extensive background) is the lack of signal. Some of the reasons are:

1. DNA/RNA degraded (check the gel by staining with ethidium bromide before transfer).

2. Transfer has not taken place. The gel can be stained after the blotting with ethidium bromide to check whether the transfer was successful. Don't be alarmed, though, if some DNA fragments, especially large ones, are still present. The gel will stain more intensely after transfer as it is very thin. If the transfer seems to be incomplete it could be due to the DNA not being denatured before transfer, the transfer buffer not being correctly prepared, short circuiting the flow of transfer solution (the stack may have collapsed to one side for example), too little buffer available for transfer.
3. The nucleic acid has not been fixed to the filter, either because baking was too short or at too low temperature, or the UV lamp was not correctly calibrated. It is important when using UV crosslinking that the filter is thoroughly dried after transfer.
4. Hybridization or washing was performed at too high temperature, in an unsuitable solution or in too large a volume.
5. The probe was not properly prepared: check percentage of incorporation with a Geiger counter if using radioactivity.
6. No homologous sequences were present in the probe. If this is suspected, repeat experiment with a positive control run on the same gel as your sample. In a northern experiment the transcript may be of low abundance. If this is suspected the following improvements for increased sensitivity are suggested:
    a. The RNA loaded on the gel should be the poly(A)$^+$ fraction rather than total RNA.
    b. Use the SSPE/formamide system, which is more sensitive than the Church protocol.
    c. Use a single-stranded probe (for example riboprobe). If a riboprobe is used in the SSPE/formamide system the washing should be performed at a higher temperature (75°C) as RNA–DNA hybrids are much more stable in the presence of formamide.
    d. Including 10% dextran sulfate or 10% polyethylene glycol in the hybridization solution will increase the effective concentration of the probe, by exclusion of nucleic acids from the volume occupied by the polymers. However the background may increase and a second disadvantage is the high viscosity of the solutions.

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CHAPTER TWENTY ONE

The polymerase chain reaction (PCR) and RT-PCR

Bruno Arcà and Charalambos Savakis

21.1 INTRODUCTION

The polymerase chain reaction (PCR), a method for the *in vitro* enzymatic amplification of DNA, has facilitated greatly the analysis of complex genomes by permitting the characterization and cloning of DNA sequences from small samples of DNA or RNA of high sequence complexity. PCR and its specialized variants are now used as widely as any of the 'older' methods in molecular biology of nucleic acids, for several reasons. First, through amplification of specific DNA sequences, or RNA sequences following reverse transcription, PCR can replace the construction of genomic or cDNA libraries for cloning. Second, PCR can simplify most analytical and synthetic procedures that follow gene cloning, such as subcloning, sequencing, synthesis of probes, mutagenesis and construction of gene fusions (Clackson *et al.*, 1991). Finally, the ability to amplify DNA from extremely small samples by PCR has revolutionized molecular genetic analysis (Erlich and Arnheim, 1992).

The PCR technique is based on repeated cycles of denaturation, annealing and DNA synthesis in the presence of specific primers flanking the region of interest, and a thermostable DNA polymerase (Arnheim and Erlich, 1992). After DNA denaturation, the two primers anneal to their complementary sequences on opposite strands of the target; the primers are arranged with their 3′ ends facing each other so that each primer extension reaction directs the synthesis of DNA across the region between the primers (Figure 21.1). In the next cycle each extension product can be used as a template for the other primer; in this way, with repeated cycles of denaturation, annealing, and primer extension, the region bounded by the two primers accumulates exponentially. Under appropriate conditions, this can result in amplification of the target sequence more than a million-fold.

Initially, DNA polymerase I Klenow fragment from *E. coli* was used for synthesis, necessitating the addition of fresh enzyme for each cycle (Saiki *et al.*, 1985). The introduction of thermostable polymerases (Saiki *et al.*, 1988) allowed automatization of the procedure through the use of thermal cycling devices, and resulted in increased specificity because of the higher temperatures used for annealing and extension.

The polymerase chain reaction can be adapted to the amplification of RNA templates (Figure 21.2). In the first step oligo-dT is used to prime the reverse transcription and produce a DNA complementary to the RNA template (random hexamers or

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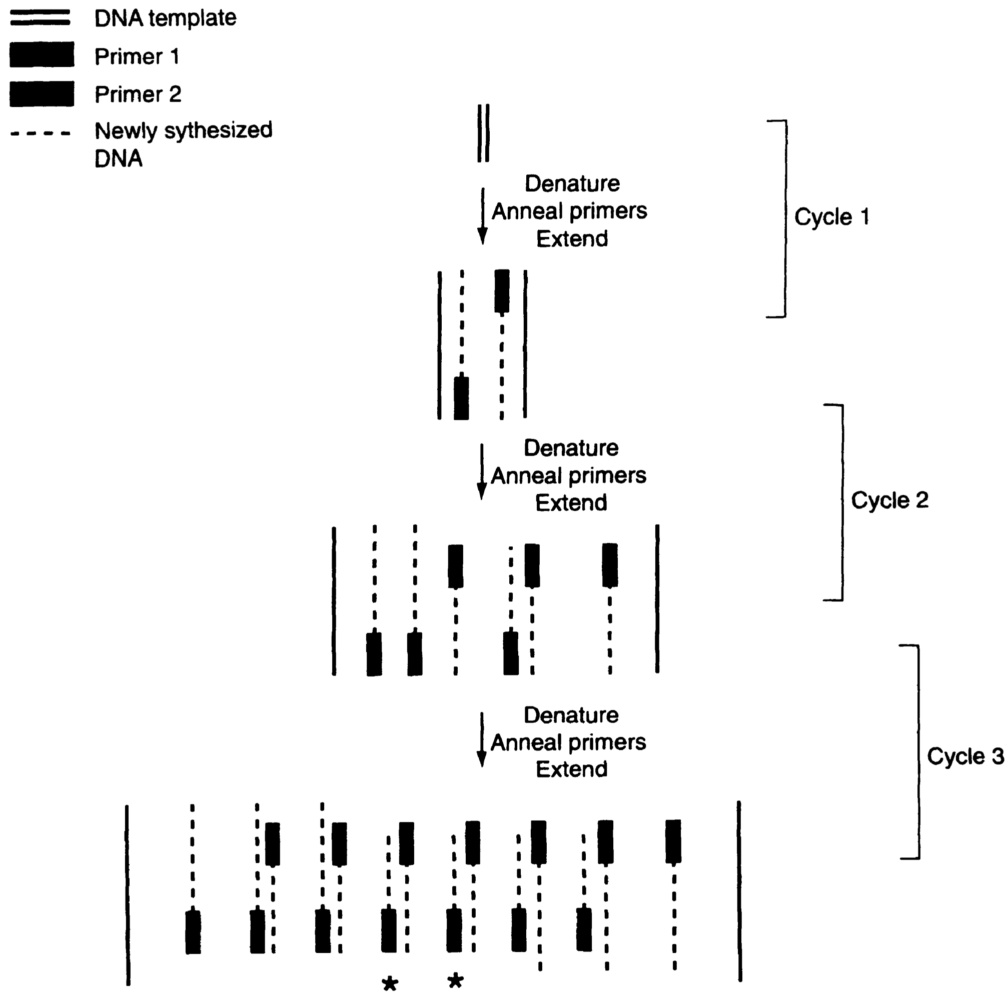


Figure 21.1 The first three cycles of DNA amplification by the polymerase chain reaction are shown. After the first few cycles the products with the ends defined by the position of the primers (marked with the asterisks) will accumulate exponentially.

specific primers can also be used to prime cDNA synthesis; Section 21.3.2); the first strand cDNA is then used in the reaction resulting in the exponential amplification of a specific cDNA. Reverse transcription PCR (RT-PCR) is a very powerful technique, both for qualitative and quantitative analysis of RNAs, and permits the amplification of full-length cDNA from rare transcripts when only a small amount of sequence information is available (Frohman *et al.*, 1988; Ohara *et al.*, 1989).

A powerful application that might be of special interest in insect disease vector studies, is the use of degenerate primers, based on conserved amino acid sequences, to clone genes or cDNAs from one species using sequence information from others that are relatively distantly related (Lee *et al.*, 1988; Robertson, 1993).

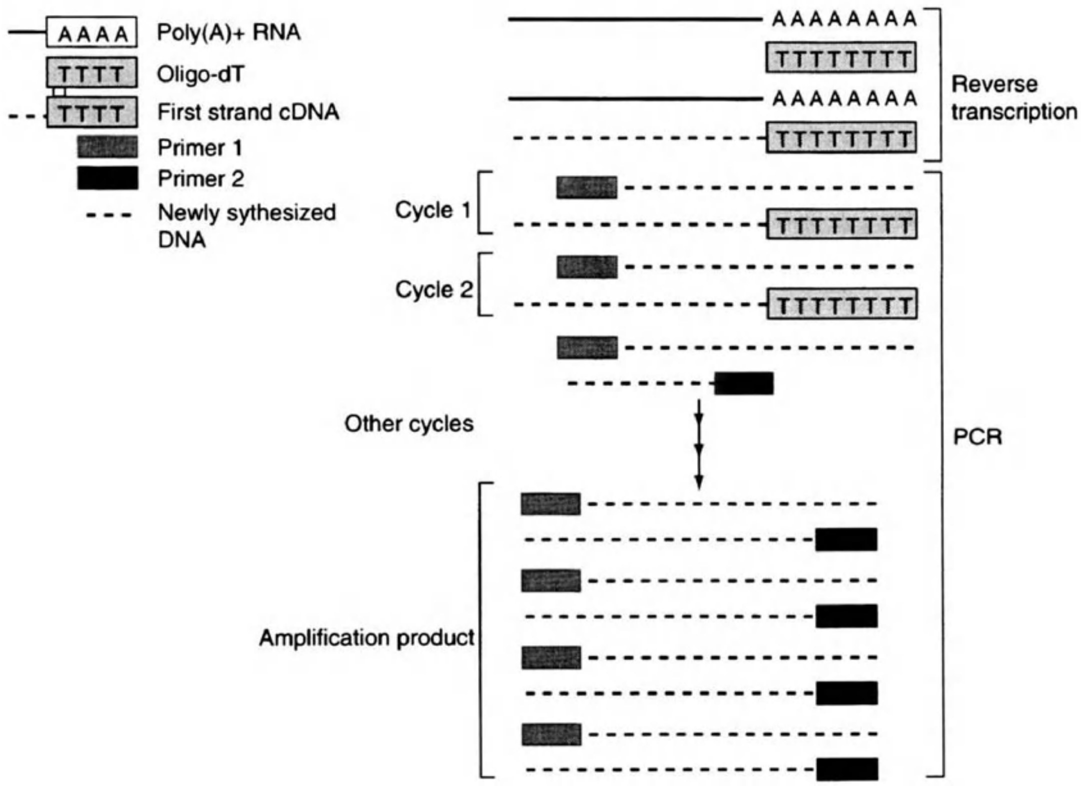


Figure 21.2 In the RT-PCR the RNA template is reverse transcribed and the first strand cDNA is then used for the amplification reaction.

Specific examples and procedures of PCR applications in insect disease vectors are presented elsewhere in this book. In this chapter we will present detailed protocols for PCR, RT-PCR, and molecular cloning of PCR products.

21.2 MATERIALS

All reagents used for PCR (as with all DNA and RNA procedures) should be of the highest grade available. The following is an exhaustive list of materials and reagents, and can be used as a checklist for setting up the procedures.

21.2.1 Purification of primers

1. Sterile H_2O.
2. *n*-Butanol.

21.2.2 Single-fly DNA preparation for PCR

1. SB (Squishing Buffer): 10 mM Tris-HCl pH 8.2, 1 mM EDTA, 25 mM NaCl. Just before use add Proteinase K to 200 μg/mL.
2. Proteinase K: 10 mg/mL in sterile H_2O. Store at –20°C.

21.2.3 Amplification reaction

1. Standard 10× Amplification buffer: 500 mM KCl, 100 mM Tris-HCl pH 8.3 (at 20°C), 15 mM $MgCl_2$, 1 mg/mL gelatin. Store at –20°C.
2. 2 mM dNTPs mix: dATP, dCTP, dGTP, dTTP 2 mM each. Dilute commercially available, neutralized 100 mM solutions with Tris-HCl 10 mM pH 7.5. Store at –20°C in 50–100 μL aliquots.
3. 100 mM $MgCl_2$.
4. PCR primers 25 pmoles/μL in sterile H_2O. Store in aliquots at –20° C.
5. Template: DNA or first strand cDNA.
6. *Taq* DNA polymerase.
7. Light mineral oil.
8. Thermo-cycler.

21.2.4 First strand cDNA synthesis

1. Template: total or poly(A)$^+$ RNA.
2. Oligo-dT_{12-18}: 100 ng/mL in TE. Store at –20°C.
3. 3 M Sodium acetate pH 5.2.
4. Absolute and 70% ethanol.
5. 400 mM KCl.
6. 100 mM PIPES pH 6.5 (adjust pH with KOH).
7. 1.25× reverse transcriptase buffer: 125 mM Tris-HCl (pH 8.3 at 42°C), 12.5 mM DTT, 10 mM $MgCl_2$, 1 mM each dNTP, 62.5 μg/mL actinomycin D, 5 mM $Na_4P_2O_7$, 0.0125% Triton-X-100. Dispense the buffer in 40 μL aliquots and store at –70°C. If the buffer is made more concentrated $MgCl_2$ and phosphate precipitate.
8. RNasin ribonuclease inhibitor 40 U/μL.
9. M-MLV reverse transcriptase 200 U/μL.
10. AMV reverse transcriptase (optional).
11. TE buffer: 10 mM Tris-HCl pH 8.0, 1 mM EDTA.
12. Phenol/chloroform: phenol:chloroform:isoamylic alcohol (50:49:1).
13. Chloroform: chloroform:isoamylic alcohol (49:1).
14. 4 M ammonium acetate.
15. 20 mM EDTA.
16. 2N NaOH.
17. STE: 100 mM NaCl, 10 mM Tris-HCl pH 8.0, EDTA 1 mM.
18. Sephacryl S-400 (Pharmacia) or Centricon-100 microconcentrator (Amicon). Optional.
19. Glycogen 20 mg/mL (mol. biol. grade).

21.2.5 Analysis and cloning of PCR products

1. Agarose. For electrophoresis of nucleic acids.
2. TBE buffer: 10× stock solution (890 mM Tris base, 890 mM Boric acid, 20 mM EDTA).
3. Ethidium bromide: 10 mg/mL in sterile H_2O. Store in the dark at 4°C. **Powerful mutagen, handle with gloves.**
4. Z-spin Plus centrifugal filters (Gelman Sciences Inc.) or equivalent.

5. 4 M LiCl.
6. Phenol: redistilled. Saturate with Tris-HCl and store in the dark at 4°C. **Avoid contact with skin and wear safety glasses.**
7. Phenol/chloroform: phenol:chloroform:isoamylic alcohol (50:49:1).
8. Chloroform: chloroform:isoamylic alcohol (49:1).
9. Glycogen 20 mg/mL (molecular biology grade).
10. Absolute and 70% ethanol.
11. TE buffer: 10 mM Tris-HCl pH 8.0, 1 mM EDTA.
12. 50 mM $MgCl_2$.
13. T_4 DNA polymerase.
14. 10× T_4 DNA ligase buffer: 500 mM Tris-HCl pH 7.5, 70 mM $MgCl_2$, 10 mM DTT. Store at –20°C.
15. 7.5 mM rATP. Store at –20°C in 10–20 μL aliquots.
16. pBCKs vector (Stratagene): digest with *Eco*RV, gel purify and resuspend in TE at a concentration of 100 ng/μL. Store at –20°C.
17. T_4 DNA ligase.
18. *E. coli* XL1-blue cells (Stratagene).
19. LB medium per L: 10 g tryptone, 5 g yeast extract, 10 g NaCl. Adjust pH to 7.0 with NaOH and autoclave.
20. LB-agar. Add to LB medium 1.5% agar and autoclave. Allow to cool to 50°C then add appropriate antibiotic.
21. 100 mM IPTG (Isopropylthiogalactoside) in sterile H_2O. Filter through 0.22 μm disposable filter. Aliquot and store at –20°C.
22. 2% X-gal (5-Bromo-4-chloro-3-indolyl β-D-galactoside) in dimethyl formamide. Store in the dark at –20°C.
23. Chloramphenicol 30 mg/mL in ethanol. Store in the dark at –20°C.

21.3 METHODS

21.3.1 PCR amplification of genomic DNA

(a) Primers

(i) Primer selection

Selection of appropriate primers is a critical, although not so predictable, parameter of a successful PCR amplification reaction; nevertheless the simple rules given below should help in the design of efficient primers.

Typical PCR primers are 20–25 nucleotides in length with a 50–60% G+C composition in order to have a T_m between 55°C and 72°C. Primers shorter than 18 nucleotides require lower annealing temperatures often resulting in nonspecific products; oligonucleotides longer than 30 are unlikely to help in increasing the specificity. A given primer pair should be balanced, both as length and G+C content, as much as possible.

Stretches of purines and pyrimidines, runs of C and/or G (three or more) at the 3′ end of the primer, can give secondary structures which may result in mispriming or prevent amplification of the target sequence and should be avoided. A pair of primers should not contain regions complementary to themselves or with each other, especially at the 3′ and 5′ ends. A complementarity at the 3′ ends may promote the formation of an artefactual product often called 'primer-dimer' and, consequently, decrease the yield of the desired product.

Potential secondary structures and complementarity can be easily revealed using one of the currently available computer programs for the design of PCR primers (e.g. 'Oligo', Rychlik and Rhoads, 1989).

(ii) Purification of primers

After the chemical synthesis and deprotection with concentrated ammonia, oligodeoxynucleotides need to be further purified before their use for enzymatic reactions. If highly purified primers are needed they can be purified by polyacrylamide gel electrophoresis (Sambrook *et al.*, 1989); in this way shorter products of the synthesis can be eliminated. We find that this time-consuming procedure is usually not necessary and that simple removal of organic contaminants results in oligonucleotides suitable for PCR amplification and even for DNA sequencing. The method described below uses *n*-butanol and is, with slight modifications, the one described by Sawadogo and Van Dyke (1991).

1. Aliquot the deprotected oligonucleotide solution in 1.5 mL microfuge tubes and vacuum-dry.
2. Resuspend one of the pellets in 100 μL sterile H_2O. (Store the rest at –20°C).
3. Add 1 mL of *n*-butanol and vortex for ~20 s.
4. Centrifuge 2–3 min at 12 000 rpm and discard the liquid phase.
5. Repeat steps 2–4 once more.
6. Vacuum-dry and resuspend in 100 μL sterile H_2O.
7. Measure optical density (1 A_{260} ~33 μg/mL) and dilute to 25 pmol/μL in sterile H_2O (1 pmol is ~6.6 ng for a 20-mer).

(iii) Primer concentration

Typical primer concentration in the amplification reaction is ~0.5 μM (i.e. 20–25 pmol in a 50 μL reaction volume) but very clean amplifications can be obtained with concentrations of 0.1 μM. In general, the specificity of the reaction drops with increasing concentration of primers. Hence, when other parameters have already been optimized and unspecific products are still present, then it is worth trying to reduce primers' concentration.

(b) DNA template

Genomic DNA to be used for PCR amplification does not need to be of high quality; successful amplifications can be obtained from relatively 'dirty' starting material such as bacterial colonies, phage plaques or crude lysates and even from partially degraded DNA preparations. On the contrary, impurities contaminating DNA preparations (e.g. traces of urea, SDS or phenol), if not sufficiently diluted, can seriously decrease the efficiency of PCR. Due to the very high sensitivity only very small amounts of target sequence (even a single copy) are necessary for PCR amplification. However, very low amounts of target DNA can increase nonspecific products and the use of 10^4 to 10^5 target copies is recommended especially when optimizing reaction conditions. For an organism with a DNA content of about 10^8 base pairs per haploid genome 10^5 target copies correspond to about 100ng of genomic DNA. Higher DNA concentrations can be successfully used but very high amounts can lead to decreased specificity.

Protocols for genomic DNA preparation have been given elsewhere in this volume (Chapter 14). We describe here a simple, rapid and reproducible procedure for iso-

lating DNA suitable for PCR from single flies (or parts of a fly) that was developed by Gloor and Engels (1992) for *D. melanogaster*. Volumes can be scaled up or down according to the size of the insect of interest and/or the number of individuals.

1. Squash the fly in a 0.5 mL tube using as pestle a yellow tip containing 50 μL of SB. Expel from the tip just a few μL of SB, smash the fly with 5–10 strokes of the yellow tip-pestle and then release the remaining SB.
2. Incubate 30 min at 37°C.
3. Inactivate proteinase K at 95°C for 1–2 min. This preparation can be stored at 4°C for a few months.
4. Use 1–5 μL in a 50 μL PCR reaction.

(c) Other reaction components

(i) Deoxynucleoside triphosphates (dNTPs)

dNTPs are used at a saturating concentration of 200 μM; higher concentrations increase the error rate of *Taq* polymerase, lower concentrations (down to 20 μM) may help in reducing priming at ectopic sites but decrease the reaction efficiency. In any case the four dNTPs should be equimolar in order to minimize misincorporation. Note that dNTPs chelate magnesium ions so, if dNTP stocks or concentrations are changed, then the optimal magnesium concentration (see below) may be affected.

(ii) Reaction buffer

Amplification buffers are usually provided by manufacturers along with thermostable DNA polymerases. A standard 10× PCR buffer contains 500 mM KCl, 100 mM Tris-HCl pH 8.3 (at 20°C), 15 mM $MgCl_2$, 1 mg/mL gelatin; some buffers include also one or more detergents (0.1% NP40, 0.1% Tween 20 or 1% Triton X-100) that seem to increase the processivity of the enzyme. 10× amplification buffers without $MgCl_2$ are also available, facilitating optimization of magnesium concentration.

(iii) Mg^{2+} concentration

Concentration of magnesium ions is a critical parameter that can influence primer annealing, DNA denaturation, primer-dimer formation and *Taq* polymerase activity; hence it may greatly affect the result of a PCR amplification. Moreover, the real free magnesium concentration is influenced by the presence of EDTA and other chelators, which may be present in the DNA preparation or in the primers, and this should be considered when using new template and/or primers or changing their concentrations. Magnesium concentrations in the range 1.5 mM to 2.5 mM are usually satisfactory for most of the primer-template combinations. However, when the amplification reaction shows low specificity or when high performances are required, then the optimal magnesium concentration should be empirically determined.

(iv) DNA polymerase

Taq polymerase is the most commonly used enzyme in PCR; its thermostability permits the enzyme to survive at the DNA denaturation temperatures (94°C) and its high polymerization activity at 72°C allows the specific annealing of the primers to their target.

Two characteristics of *Taq* polymerase have an important practical consequence. The first is the absence of proofreading activity that results in a misincorporation rate of about 10^{-4} nucleotides/cycle. When accurate sequence information is needed and many cycles of amplifications are necessary (e.g. with low amounts of starting template), then direct sequencing of the PCR fragment or sequencing of several individual clones might be necessary. A possible alternative is the use of commercially available polymerases with proofreading activity such as *Pfu* from *Pyrococcus furiosus* or *Vent* from *Thermococcus litoralis*. The second characteristic is the tendency of *Taq* polymerase to add a 'tail' of a single nucleotide (preferentially an A) to the 3′ ends of a blunt-ended duplex DNA fragment. Such activity can significantly decrease the efficiency of blunt-end cloning of PCR products (see below).

For most PCR applications, between 1 and 2.5 U of *Taq* polymerase per 100 μL reaction are recommended; higher concentrations of enzyme can sometimes increase the yield of unspecific products. Because *Taq* polymerase is not indefinitely resistant to heat, some protocols suggest adding the enzyme after the initial 5 min denaturation step. However, especially when processing large batches of samples, we find that it is more accurate, reproducible and convenient to add the enzyme to a fresh master mix prepared for a number of reactions.

(v) Additives

Sometimes standard PCR conditions do not work with the expected efficiency and/or specificity on target DNA with high G+C content or with complex secondary structures. In some of these cases the inclusion in the reaction buffer of additives can positively affect the PCR amplification results. For example, the addition of DMSO at a final concentration of 5–10% can be a decisive factor in the amplification of fragments containing secondary structures (Shen and Hohn, 1992) while the use of formamide (1–5%) has been reported to significantly improve specificity when amplifying G+C-rich segments (Sarkar *et al.*, 1990). Also the addition of 5–20% glycerol to the PCR buffer has been reported to increase both the yield and the specificity of amplification reactions that work inefficiently under standard conditions (Lu and Negre, 1993).

(d) Cycling parameters

As previously described, a typical PCR cycle consists of three steps (denaturation, annealing and extension) with different requirements for temperatures and times. As a general rule, the minimum effective amount of time should be used at each step in order to 'save' enzyme and improve the efficiency of the reaction.

1. Denaturation is a critical step because incomplete strand separation of the target template and/or the PCR product can result in the failure of your PCR experiment. Usually 1 min denaturation at 94°C at the beginning of the cycle is effective for most samples; however targets with very high G+C content or strong secondary structure may require higher temperatures (95–97°C). When raising temperature consider that *Taq* polymerase half-life decreases dramatically at higher temperatures, being 40 min at 95°C but only 5 min at 97.5°C (Gelfand and White, 1990).
2. Annealing temperature depends on the T_m (melting temperature) of the primers but needs empirical determination. Primers that work at annealing tempera-

tures in the range 55–72°C usually give the best results. The T_m of a primer can be calculated with the simple formula:

$69.3 + 0.41(\%G+C) - 650/L$

(L = primer length) (Mazars *et al.*, 1991)

Optimization of the annealing temperature can be conveniently achieved with 2–5°C increments/decrements in subsequent optimization runs. Stringent temperatures, specially in the initial cycles, will help increase the specificity. Because primers are used in large excess their annealing occurs quickly and annealing times of 30–60 s are effective in most cases.

3. Extension is traditionally performed at 72°C, a temperature close to the optimal for *Taq* polymerase (~75°C). However, *Taq* polymerase is active at lower temperatures and a simple two-step cycle (94°C denaturation, 60–70°C annealing and extension) also gives excellent results. The extension time mainly depends on the length of the sequence to be amplified and the use of 1 min per kb of target is more than enough. Extension times of 30 s are usually sufficient for products up to 500 bp.
4. Cycle number depends mainly on the copy number of target template when other parameters are optimized. If the number of target copies is 10^4–10^5 then 30 cycles should be enough to visualize the product on an agarose gel; 35–45 cycles might be necessary with a lower copy number.

(e) Amplification reaction

1. Prepare two sterile 0.5 mL tubes. Add to the first tube 5 μL of sterile H_2O and to the second one the template DNA (10^4–10^5 target copies) in a final volume of 5 μL. The reaction performed without template is a useful control and permits the reaction components to be tested for possible contamination with the target sequence.
2. In a sterile tube prepare a two-reactions cocktail mixing in the following order:

Sterile H_2O	65.5 μL
Standard 10× amplification buffer	10 μL
2 mM dNTPs mix	10 μL
Primer 1 25 pmol/μL	2 μL
Primer 2 25 pmol/μL	2 μL
Taq DNA polymerase 5 U/μL	0.5 μL

3. Add 45 μL of the cocktail to each tube prepared in step 1.
4. To prevent evaporation overlay the reaction mixture with one or two drops of light mineral oil.
5. Typical amplification conditions for 20-mer primers with a 50% G+C content and a target less than 1 kb in length are as follows:
   Initial denaturation: 5 min at 94°C.
   30 cycles: 1 min at 94°C, 1 min at 55°C, 1 min at 72°C.
   Last cycle: 1 min at 94°C, 1 min at 55°C, 10 min at 72°C.
   End: 4°C.

   The initial denaturation step of 5 min at 94°C is usually performed before starting the cycles both to ensure full denaturation of complex DNA templates and to inactivate proteases or nucleases eventually present in the sample. The final extension step of 5–10 min at 72°C is suggested in the attempt to obtain as many blunt-ended products as possible.
6. After the reaction is complete, analyse a 10 μL aliquot of the amplified DNA on an agarose gel. The use of agarose with high resolution power (3% NuSieve/1%

SeaKem, FMC Bioproduct) can be useful in the analysis of PCR products from 100 bp to ~1 kb. The mineral oil can be removed by just spotting the reaction on a piece of parafilm (the oil tends to stick to the parafilm and the aqueous solution can be easily recovered).

7. Examine the ethidium bromide stained gel to control the yield and specificity of the reaction. The product should appear as a predominant sharp band of the expected size. Additional minor bands due to nonspecific priming, single-strand products (if the primers are present in unequal molar amounts) and the primers themselves might be visible as well.

We suggest trying this protocol first with the standard magnesium concentration of 1.5 mM; if results are unsatisfactory then the $MgCl_2$ concentration needs to be optimized. This is not worth doing when one or just a few samples have to be analysed and the reaction has not to be routinely repeated. In the other cases the optimal $MgCl_2$ concentration should be determined dispensing aliquots of a magnesium-free master mix into tubes containing $MgCl_2$ concentrations ranging from 0–6 mM with 0.5–1.0 mM increments.

When sequence information on the expected product is available then confirmation that the PCR product is the desired one can be obtained for example: (i) by restriction enzyme analysis if a restriction endonuclease is known to cut within the product; (ii) using a third internal primer, in combination with one of the first pair, in a re-amplification reaction; (iii) by Southern hybridization. However sometimes this is not possible and cloning and sequencing (or direct sequencing) of the PCR product is necessary.

Sometimes a second round of PCR amplification is necessary in order to obtain preparative amounts of the fragment of interest; in these cases, it is preferable to use as template the amplified fragment after gel purification to avoid concomitant reamplification of minor nonspecific products. The simplest way to do this is to take a small piece of agarose containing the band of interest with a pipette tip, place it into a tube with fresh PCR cocktail, and reamplify.

21.3.2 PCR amplification of RNA (RT-PCR)

Methods for preparation and handling of RNA are described in Chapter 15 and further details can be found in several laboratory manuals (Sambrook *et al.*, 1989; Ausubel *et al.*, 1991) so they will not be discussed here. The RNA template used in the reverse transcription step can be total, cytoplasmic or poly(A)$^+$ RNA and the choice depends on quantities available and rarity of the mRNA according to the specific application. Purified poly(A)$^+$ RNA is of course the best template and its use is especially suggested for rare or large mRNAs; however total RNA usually gives more than satisfactory results.

The primer for the reverse transcription reaction can be oligo-dT, random hexamers or a primer specific for the mRNA target. Priming by oligo-dT most frequently produces full-length cDNAs; random hexamers produce partial cDNAs but they have been reported to be more efficient (Kawasaki, 1990) and their use might help in overcoming difficulties caused by template secondary structures. Specific cDNA primers, instead, will selectively extend the mRNA of interest; however, when designing specific cDNA primers, consider that oligonucleotides too rich in their

G+C content produce more nonspecific reverse transcription so they will increase the overall background of the amplification.

The protocol described here uses oligo-dT and consists of three steps: (i) annealing of the cDNA primer to the RNA (ii) reverse transcription and (iii) amplification of the synthesized first strand cDNA. The first strand cDNA synthesis reaction is performed, with few modifications, as described by Brown and Kafatos (1988). This protocol was developed for the production of long cDNAs and gives satisfactory results with cDNAs longer than 5 kb.

1. In a 1.5 mL sterile microfuge tube mix:

Total or poly(A)$^+$ RNA	variable amount
oligo-dT$_{12–18}$	100–200 ng (~20–40 pmol)
Sterile H_2O	to 90 μL

   The amount of RNA template used in the reverse transcription reaction may vary from 1 up to 50 μg of total RNA or from 100 ng to 2 μg of poly(A)$^+$ RNA. At least 1 μg of total RNA or 25 ng of poly(A)$^+$ RNA should be used, after reverse transcription, in the PCR amplification reaction. Add 10 μL 3M Na-acetate pH 5.2 and 200 μL ethanol. Mix then leave at –70°C for 15 min. This co-precipitation step maximizes the efficiency of the annealing. 5–100 pmol of random hexamers or cDNA specific primers can be used in place of oligo-dT.
2. Centrifuge at 4°C for 15 min at ~13000 **g**.
3. Wash the pellet with 70% ethanol, centrifuge 2–3 min at full speed and air-dry.
4. Dissolve the pellet in 7 μL of 200 mM KCl, 5 mM PIPES pH 6.5.
5. Incubate at 68°C for 5 min then leave to cool slowly to 55°C and finally chill on ice. This can be best done by incubating the microfuge tube in a metal rack at 68°C for 5 min then taking the rack out of the heater and leaving it on the bench to slowly cool to 55°C.
6. To the 7 μL annealed RNA–cDNA primer add:

1.25× reverse transcriptase buffer	40 μL
RNasin 40 U/μL	1 μL
M-MLV reverse transcriptase 200 U/μL	1 μL
AMV reverse transcriptase 20 U/μL	1 μL

   Incubate 1 h at 40°C

   The use of both M-MLV and AMV reverse transcriptases increases the yield in long cDNAs (Brown and Kafatos, 1988); the two enzymes seem to pause at different sequences so partial transcripts of one enzyme can be extended by the other. For average sized cDNA synthesis the use of only one of two enzymes may be sufficient.
7. At the end of the incubation period add 50 μL of TE then extract once with 1 vol of phenol/chloroform and once with 1 vol of chloroform.
8. Add 100 μL of 4 M ammonium acetate and 400 μL of ethanol.
9. Leave 15 min at –70°C then proceed as in steps 2 and 3.
10. Resuspend in 5 μL of 20 mM EDTA then add 5 μL of 0.2 N NaOH.
11. Incubate 15 min at 65°C then add 90 μL of STE. During this step RNA is hydrolysed. At this point, if the cDNA primer is also one of the amplification primers, then proceed to step 12; otherwise the cDNA primer should be removed by running the cDNA through a spun column of Sephacryl S-400 (Sambrook *et al.*, 1989); a possible alternative to Sephacryl is the use of a Centricon-100 microconcentrator according to the manufacturer's instruction.

12. Add 100 μL of 4 M ammonium acetate, 1 μL of 20 mg/mL glycogen and 500 μL of ethanol.
13. Leave 15 min at –70°C then proceed as in steps 2 and 3.
14. Resuspend the pellet in 1 μL of sterile water for each μg of starting total RNA (or every 25 ng of starting poly(A)$^+$ RNA).
15. Assemble the PCR amplification reaction adding 1–5 μL of the first strand cDNA to the following PCR cocktail:

10× amplification buffer	5 μL
2 mM dNTPs mix	5 μL
Primer 1 25 pmol/μL	1 μL
Primer 2 25 pmol/μL	1 μL
Sterile H_2O	to 50 μL
Taq polymerase	1–2.5 U

16. Overlay with one or two drops of mineral oil and amplify.

An initial denaturation step of 5 min at 94°C should be included to destroy any possible secondary structure. Cycling parameters, as for annealing temperature and extension time, depend on the T_m of the primers and the length of the fragment to be amplified as previously discussed. The number of cycles depends on the abundance of the target; 40 cycles should be sufficient for rare mRNAs if the starting template is poly(A)$^+$ RNA. When the number of target copies is low a second round of amplification might be necessary; in such cases, especially if the result of the first amplification is a smear, the use of nested primers for the reamplification can be very beneficial. As general rule, an increased specificity can be obtained if the downstream primer in the amplification reaction is different from the cDNA primer.

Due to the very high sensitivity of the PCR reaction, the possibility that the amplified product could have originated from small amounts of genomic DNA contaminating the RNA preparation should be considered. Whenever possible, primers for RT-PCR amplifications should be designed in different exons, so products from DNA or cDNA can be easily distinguished. When this is not possible samples should be treated with RNase-free DNase before the reverse transcription reaction (followed by phenol extraction and ethanol precipitation) and appropriate controls should be included in the amplification reaction. Suggested controls: (i) no template; (ii) no reverse transcribed RNA; (iii) DNase-treated and (iv) DNase-untreated reverse transcribed RNA.

21.3.3 Cloning PCR products

As previously mentioned, blunt-end cloning of PCR products can be complicated by the tendency of *Taq* polymerase to add an extra nucleotide (commonly A) at the 3′ end of a blunt DNA fragment. Different strategies can be used to overcome this problem for example (i) the design of primers with restriction sites at their 5′ ends or (ii) the use of the so called T-vectors (Marchuck *et al.*, 1991; Holton and Graham, 1991). The protocol described below uses the 3′–5′ exonuclease activity of the T_4 DNA polymerase to remove these extra 3′ bases before the cloning step and relies on blue-white selection for the recovery of recombinant clones (Sambrook *et al.*, 1989).

1. Precipitate the amplification reaction remaining after agarose gel analysis (usually 90 μL) with 2.5 M ammonium acetate and 2.5 vol of ethanol. Leave at –70°C for 15 min.
2. Centrifuge at ~13 000 g for 15 min at 4°C.
3. Wash the pellet with 70% ethanol, centrifuge 2–3 min then dry and resuspend in 10–20 μL TE.
4. Run the sample on a 1% agarose gel.
5. Using a sterile razor blade cut the DNA band, as small as possible, out of the gel. Use a long-wave UV lamp (330–360 nm) to minimize nicks in your DNA.
6. Load the gel slice into a 0.5 mL Z-Spin filter and incubate for 5 min in liquid nitrogen.
7. Centrifuge for 15 min at ~13 000 g. The agarose matrix, disrupted by the quick freezing, is retained on the filter whereas the aqueous solution containing the DNA is recovered in the filtrate.
8. Add 1/10 vol of 4 M LiCl, then extract once with phenol, once with phenol-chloroform and once with chloroform.
9. Add 1 μL of glycogen 20 mg/mL as carrier and 2.5 vol of ethanol. Use of glycogen is specially suggested when precipitating small fragments and/or small amounts of DNA.
10. Incubate at –70°C for 15 min then centrifuge as in step 2.
11. Wash the pellet with 70% ethanol, centrifuge 2–3 min then dry and resuspend in 5 μL of TE.
12. To the 5 μL of purified PCR fragment add in the following order:

Sterile H_2O	2 μL
50 mM $MgCl_2$	1 μL
1 mM dNTPs mix	1 μL
T_4 DNA polymerasel U/μL	1 μL

13. Incubate 15 min at 12°C then inactivate T_4 polymerase at 75°C for 10 min. The Klenow fragment of *E.coli* DNA polymerase I is also commonly used for this purpose; however, T4 DNA polymerase seems to be much more efficient (Costa and Weiner, 1994).
14. Set up the ligation reaction adding to the 10 μL of blunt-ended PCR product:

*Eco*RV digested pBCKS 100 ng/μL	1 μL
10× T_4 ligation buffer	1.5 μL
rATP 7.5 mM	1 μL
T_4 DNA ligase 1 Weiss U/μL	1.5 μL

Incubate from 4 h to overnight at 18°C. In this ligation reaction the molar ratio between PCR fragment and cloning vector can be as high as 10:1 or even more (according to the size of the fragment and the amount of DNA recovered). This does not create any problem of concatamerization because PCR primers do not have a phosphate group at their 5′ ends. Always set up as control a self-ligation of the vector alone to evaluate the background of white colonies; the number of white colonies in the ligation containing the PCR product should show at least a five-fold increase.

15. Use 2.5 μL of this ligation mixture to transform XL1-blue competent cells prepared with the Hanahan method (Hanahan, 1985 ; Sambrook *et al.*, 1989) so to have an efficiency of at least 10^7 colonies/μg DNA.
16. Plate on LB plates containing 30 μg/mL chloramphenicol. To avoid overcrowded plates spread 100–200 μL (out of 1 mL) per plate. For colour selection

of recombinant clones 40 μL of 100 mM IPTG and 100 μL of 2% X-gal should be spread on the plates about 30 min before plating (do not mix IPTG and X-gal together, they will precipitate).

17. Incubate plates at 37°C for about 15–18 h. At the end of the incubation at 37°C leave the plates for a few hours at 4°C; this increases the blue colour and allows a better selection of the white colonies. The chloramphenicol resistant pBCKS vector can be substituted with the more commonly used ampicillin resistant vector pBSKSII or any other vector that permits white–blue colour selection.
18. Using sterile toothpicks inoculate 6–12 white colonies in LB containing 30 μg/mL chloramphenicol. Grow overnight for plasmid DNA minipreparations and restriction enzyme analysis. A typical result is that between about 50% and 90% of the white colonies are recombinant.

The procedure described above works consistently and reproducibly for PCR products from ~200 bp to ~1.5 kb and avoids manipulations like dephosphorylation of the vector and phosphorylation of the fragment (oligonucleotide primers usually do not have a phosphate group at their 5′). The selection of white colonies in the high background of blue colonies is facilitated by plating from 1/10 to 1/5 of the transformation mixture per plate.

For fragments longer than 1.5 kb the efficiency of the method might be low, especially if the fragment is not in high molar excess in the ligation reaction. In these cases and when a PCR product is resistant to cloning (for unknown reasons some PCR products are very difficult to clone) alternative strategies, such as the previously mentioned T-vectors, should be considered.

NOTES

What can go wrong?

1. No amplification. The failure of a PCR amplification may depend on one of several variables and the most obvious ones, template and primers, should be considered first. Integrity of genomic DNA (or RNA used for reverse transcription) can be controlled running a sample onto an agarose gel; DNA or cDNA template can be functionally tested using a pair of primers from a previously standardized PCR amplification. Sequence and concentration of primers should be carefully checked. If template and primers look good then it becomes necessary to change the reaction conditions decreasing the annealing temperature (Section 21.3.1d) and/or optimizing magnesium chloride concentration (Section 21.3.1e); for some targets the addition of DMSO, formamide or glycerol might be helpful (Section 21.3.1c). If there is still no improvement and you are convinced about the quality of the other reaction components (dNTPs, buffer and *Taq* polymerase) then it may be worth designing a new pair of primers; keep in mind that some primer pairs just do not work.
2. Unspecific amplification. In many cases secondary bands, smears and other artefacts may appear, along with the expected product, on the agarose gel. This is usually due to unspecific priming and may represent a problem if the yield of the specific product is seriously affected and/or a perfectly optimized reaction is required. Generally lower primer concentration, fewer cycles and increased annealing temperatures help in increasing the specificity. When a reaction

shows a low specificity we suggest, first, increase the annealing temperature to the maximum possible and then, if results are still unsatisfactory, optimize the magnesium concentration (Section 21.3.1e) and eventually lower the concentration of the primers (Section 21.3.1a). However, sometimes the highest specificity can be obtained only with the 'hot start' technique. This is based on the assumption that most of the nonspecific annealing and false primer elongation events occur when all the reaction components have been mixed (usually at room temperature) and before the initial denaturation step. In the 'hot start' method at least one of the essential reaction components is excluded from the reaction mixture until the temperature is high enough to suppress false primer annealing. In the manual 'hot start' *Taq* polymerase is excluded from the initial mixture and is separately added to each tube only after the temperature reaches 70–80°C. In the automated procedure a layer of paraffin wax keeps the mixture and the omitted reagents physically separated and permits their mixing only at high temperature during the initial denaturation step (Bassam and Caetano-Anolles, 1993).

3. Contamination. Due to the very high sensitivity of the polymerase chain reaction contamination can be or become a serious problem; false positives can result from sample cross-contamination but the most dangerous source of contamination is the carry-over of DNA from previous amplification of the same target. The simple procedures listed below help to minimize the risk of contamination:
   a. Perform the assembly of PCR reactions and post-PCR analysis in two physically distinct areas of the lab.
   b. Keep a set of pipettes only for PCR reactions and use aerosol-free tips. Alternatively use positive displacement pipettes.
   c. Store PCR reagents in small aliquots (so in case of contamination they can be simply discarded and you can start immediately with a new set).
   d. When assembling PCR reactions wear gloves and change them often.
   e. Briefly centrifuge tubes containing PCR reagents before opening them.
   f. Always include a 'no template' control reaction.

Some other useful suggestions about avoiding contaminations can be found in Kwok and Higuchi (1989). If contamination problems arise then you might subject the PCR cocktail (before adding DNA template and enzyme) to (i) UV irradiation and so crosslink contaminating target sequences (Sarkar and Sommer, 1990), or (ii) DNAase treatment (Furrer *et al.*, 1990). If the carry-over contamination has become an acute problem then design a new set of primers, externally to the original pair, and consider the possibility of using one of the commercially available carry-over prevention kits (Longo *et al.*, 1990).

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CHAPTER TWENTY TWO

Differential display of mRNA

George Dimopoulos and Christos Louis

22.1 INTRODUCTION

Most biological processes rely on changes in the level and pattern of gene expression. Their molecular and functional dissection often requires the isolation and subsequent characterization of genes that are differentially expressed in space (different tissues or organs), in time (different developmental stages), or in only one sex. Only 15% of the genes are expressed in all cells, and even fewer are constitutively expressed exhibiting no temporal specificity at all. Until recently, there were only two alternative approaches for the isolation of differentially expressed genes: differential hybridization using various probes, or the use of subtractive cDNA libraries. Both methods are time-consuming and require large amounts of starting material, especially for the construction of the libraries.

These problems have been overcome by the development of a new PCR-based method which relies on the amplification of reverse transcribed mRNAs, originating from different sources to be compared to each other. The method uses random primers and in analogy to the RAPD technique on which it is practically based, one could call it Random Amplified Reverse Transcribed RNA; it is, though, generically known by the term Differential Display of mRNA. This technique allows the easy and rapid detection (and subsequent isolation) of differentially expressed mRNAs, using for comparison purposes two or more mRNA-pools, one of which is presumed to contain the mRNA to be identified and the other(s) not. This chapter will describe a method that does not rely on the use of radioactive precursors for the identification of the amplification products. In addition to the obvious advantage of working with safer material, the protocol is faster than the 'radioactive' one at no substantial loss of resolution.

22.1.1 Differential display in theory

The method involves three basic steps: (i) cDNA synthesis, (ii) PCR-amplification of the cDNAs using random primers, and (iii) resolution of the PCR products by gel electrophoresis. Several variations have been described for the different steps, each one with its specific advantages and disadvantages. In some protocols the first two steps are connected (done in the same reaction tube; Welsh *et al.*, 1992) while in

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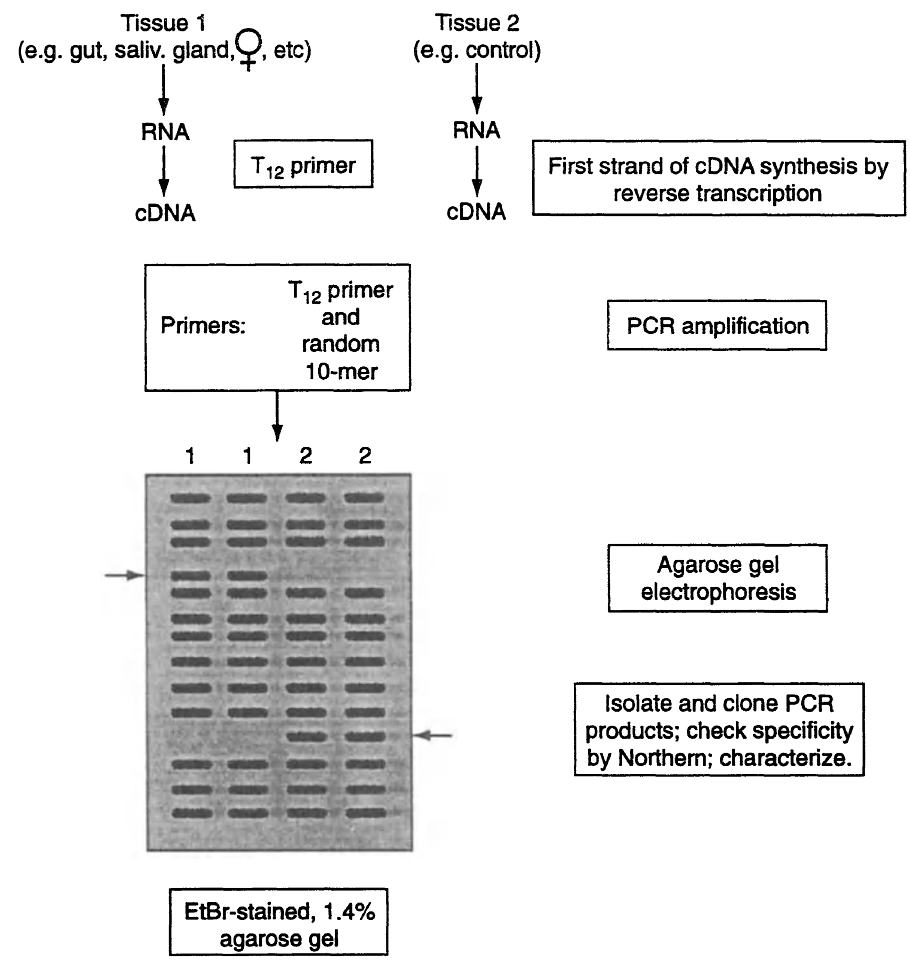


Figure 22.1 The figure shows a schematic representation of the differential display method as described here. The different steps are indicated in the boxes on the right. The arrows on the 'gel' indicate two cDNAs that are differentially displayed in the two tissues compared, 1 and 2.

others they are separated (Sokolov and Prockop, 1994). Several factors should be considered for the design of the experimental approach. They include: (i) the availability of biological material; (ii) the number of expected, differentially expressed genes; (iii) the abundance of differentially expressed mRNAs; (iv) the length of amplified cDNAs one wishes to obtain.

(a) Step 1. cDNA synthesis

The reverse transcription of the mRNA can either be primed with a dT_{10-12} primer or with a random (6–20 b long) primer. When the starting material for the cDNA synthesis is total RNA, an oligo(dT) primer is usually preferred such that poly(A)$^+$ RNA can be selectively used as a substrate (Liang and Pardee, 1992). In contrast, when the starting material consists of already oligo(dT)-selected mRNA, the first strand cDNA synthesis is usually performed with an arbitrary primer of 20 b (Welsh *et al.*, 1992) or even with fully degenerate hexameric primers (Sokolov and Propkop, 1994). The

advantage of using random primers is that fragments of the more informative internal coding sequences of the mRNAs can be amplified in the subsequent PCR step, instead of the less informative 3′ untranslated sequences, which the oligo-T primer will have a bias for. In the method described by Liang and Pardee (1992) dT_{11} primers with an additional two nucleotides of the 12 possible combinations on their 3′ end (i.e. $5'T_{11}CA\text{-}3'$) were used in order to subdivide the total cDNA into 12 fractions with nearly equal numbers of represented mRNA species. This generated less complex fingerprints on the gels used to resolve the PCR-amplified products.

(b) Step 2. PCR amplification of the cDNAs

In the first PCR cycle the arbitrary primer will anneal to a complementary sequence potentially present in only a fraction of the cDNAs synthesised, and the second strand synthesis will proceed in the direction corresponding to the 3′ end of the mRNA. In the subsequent cycles the selected cDNAs will get amplified. The PCR amplification employs the primer used in the previous step (reverse transcription) in combination with the second, arbitrary primer. In the case where the first strand synthesis was primed with degenerate hexameric primers, usually two or more (10–22 b long) arbitrary primers are used (Sokolov and Prockop, 1994). By using several (>2) primers in different reactions, a larger number of individual cDNAs can, thus, be amplified and more complex fingerprints will be generated on the gel. The PCR amplification can be performed with radioactive (^{32}P or ^{35}S) or nonradioactive

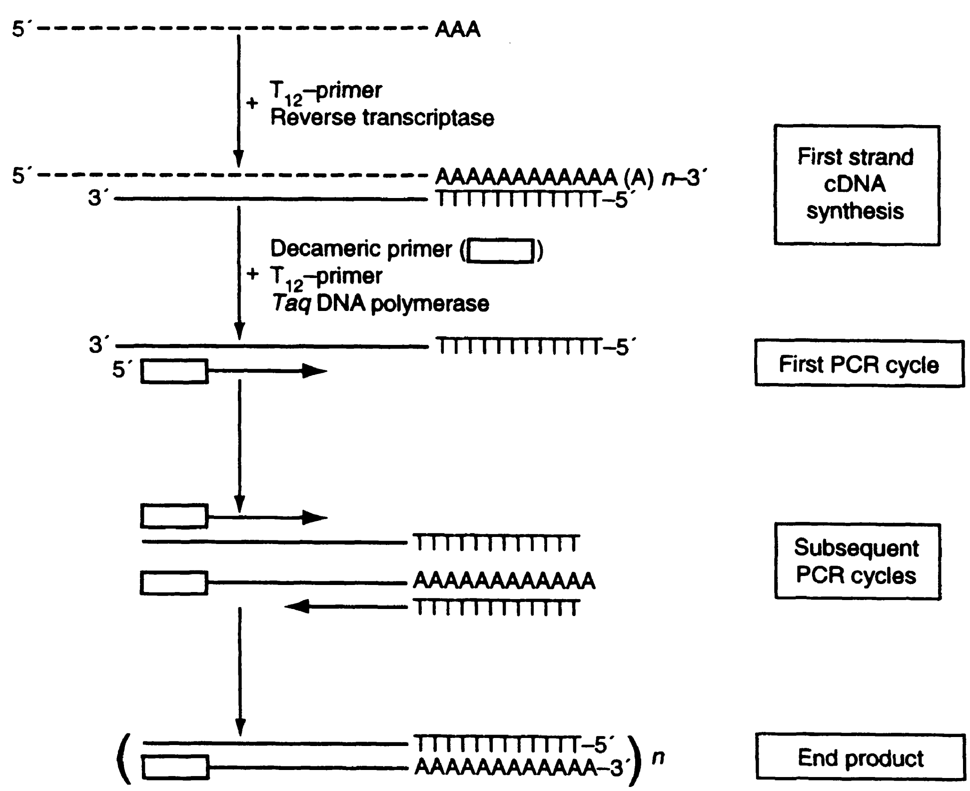


Figure 22.2 The figure schematically describes the reactions which lead from mRNA to the amplified cDNA.

nucleotides, depending on the type of gel to be used to separate the products. The temperature profiles of the PCR cycles have to be determined from the type of primers used for amplification.

(c) Step 3. Resolution of PCR products

The differential display products can be resolved either on acrylamide 'sequencing' gels if radioactive precursors were used, or agarose gels in the opposite case. The sequencing gels have a higher resolution, since up to 150 bands can be resolved on a single lane. Furthermore, since the products are radioactively labelled, less abundant mRNAs can be easily detected (Liang and Pardee, 1992; Bauer *et al.*, 1993). The main drawbacks of resolving the products on sequencing gels are: (i) only relatively small fragments of a narrow size range window (50–500 bp) are resolved; (ii) the isolation of the fragments for further analysis is tedious (e.g. precise aligning of autoradiogram and the dried acrylamide gel) and routinely requires re-amplification; (iii) 'hot' material has to be employed in the PCR and can potentially be a source of radioactive contamination. Fewer fragments (<50) but, in contrast, of a wider size range (100–2000 bp) can be resolved on agarose gels (Sokolov and Prockop, 1994). The fragments can be directly excised from the gel for cloning using any standard protocol, and no radioactivity is involved, making the whole procedure faster, easier and safer.

Each step of the method is dependent on the other. For example, if the reactions are to be resolved on an agarose gel the first two steps may have to be designed in such a way as to generate high yields of a relatively low number of fragments (Sokolov and Prockop, 1994).

22.1.2 Differential display in practice

The differential display protocol presented below describes a variation of the method where the reverse transcription of RNA and the subsequent PCR-amplification of the cDNA are performed in two separate steps. First, dT_{12}-primed total RNA is reverse transcribed into first strand cDNA; then, the cDNA is PCR-amplified using a random decameric primer and the dT_{12} primer. Nonradioactive nucleotides are used for the PCR step and the reaction products are resolved on a 1.4% agarose gel (Figures 22.1 and 22.2). This protocol is especially suitable when limited amounts of tissue are available as is often the case with vector insects and, thus, purification of poly(A)$^+$ RNA is not possible. It has been successfully used for the isolation of sex- and tissue-specific genes from the malaria vector *An. gambiae*.

22.2 MATERIALS

22.2.1 cDNA synthesis

1. Phenol: Re-distil phenol at 160°C. Equilibrate the phenol by repeated extractions with 50 mM Tris, pH 8.0, until the pH is above 7.6. Add 8-hydroxyquinoline as an antioxidant and store at 4°C.
2. Chloroform/isoamyl alcohol: Mix them in a ratio of 24:1. Store at RT.
3. Potassium acetate stock solution: (3 M, pH 7.5). Store at RT.
4. 10× RT-buffer: 500 mM Tris-HCl (pH 8.3), 500 mM KCl, 40 mM $MgCl_2$.
5. dNTP stock: 10 mM each of dATP, dCTP, dGTP and dTTP in H_2O. Adjust to pH 7.0 with 100 mM NaOH using pH paper. Store at –20°C or lower.

6. DTT stock: 0.1 mM in H_2O. *Do not autoclave!* Sterilize by filtration and store aliquots at –20°C.
7. dT_{12}-primer: 0.1 mM in H_2O. Store at –20°C.
8. M-MLV reverse transcriptase (Pharmacia 20 U/μL in H_2O). Store at –20°C.
9. RNasin (Promega 40U/μL in H_2O). Store at –20°C.
10. Prepacked Sephadex G-25 column (Pharmacia).
11. Diethylpyrocarbonate (DEPC)-treated H_2O: DEPC readily degrades to Ethanol and CO_2. Use DEPC from a bottle that was only recently opened. *Open bottle in the fume hood.* Store at 4°C. To treat the water add 10 mL of DEPC/L of H_2O. Let stand for 24 h and autoclave. Use this water for buffers only after the DEPC has been destroyed by autoclaving.

22.2.2 PCR-amplification

1. 10× PCR-buffer: 100 mM Tris-HCl, pH 9.3, 500 mM KCl, 1% Triton-X100.
2. $MgCl_2$: Use aliquots of a concentration of 10 mM from a stock solution of 1 M. Autoclave and store the aliquots at –20°C.
3. dNTP stock: 2 mM each of dATP, dCTP, dGTP and dTTP in H_2O. Store at –20°C.
4. Random sequence decameric primer: (Operon Tech. 10 mM in H_2O, or BC). Store at –20°C.
5. Oligo-T(12) primer: 10 mM in H_2O. Store at –20°C.
6. *Taq* DNA Polymerase (Promega 5 U/μL in H_2O). Store at –20°C.
7. Mineral oil for PCR (Sigma).

22.2.3 Gel electrophoresis

1. Use a 1.4% agarose gel. Stain with ethidium bromide (0.5 μg/mL from a 10 μg/mL stock solution stored in the dark) for 30 min, destain in H_2O. *Wear gloves! Ethidium bromide is a strong mutagen.*
2. DNA 'loading buffer': 0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in H_2O (or your own preferred loading buffer). Store at –20°C.

22.3 METHOD

22.3.1 cDNA synthesis

Several methods for RNA extraction have been described and a number of commercial RNA extraction kits are available on the market. Some of them are specially designed for a certain type of organism or tissue. For the extraction of RNA from mosquitoes, we have been using a simple protocol that results in high yields of intact RNA (Noriega and Wells, 1993). Although not tested, most standard methods, including the ones described in Chapter 15 should work equally well.

1. Using your favourite protocol, isolate total RNA from the sources that you want to use for your comparisons. ~10 μg of total RNA will be used for the cDNA synthesis. This will yield enough material for several PCR reactions (~50).
2. Ethanol precipitate and pellet RNA by centrifugation at maximal speed in a microfuge tube for 10 min. Remove the supernatant and dry the pellet in a desiccator.

3. Redissolve the RNA pellet in 10 µL (or more) DEPC-treated H_2O and estimate the concentration on a gel or by spectrophotometer (Sambrook *et al.*, 1989); adjust to 1 mg/mL with DEPC-treated H_2O. Store at –200°C until further use.
4. Incubate 10 µg RNA in 10 µL DEPC-treated H_2O at 65°C for 5 min.
5. Add the following mix to the RNA at 37°C:
   25 µL H_2O
   5 µL 10× RT buffer
   2.5 µL dNTPs
   2 µL DTT
   3 µL dT12 primer
   2 µL M-MLV reverse transcriptase
   0.5 µL RNasin
6. Incubate at 37°C for 1 h. Stop the reaction by heating to 65°C for 10 min to inactivate the reverse transcriptase. Immediately put on ice.
7. Pass the reaction through a Sephadex G-25 column according to the manufacturer's instructions to remove the reaction components.
8. Add H_2O to a final volume of 100 µL and store at –20°C until further use.

22.3.2 PCR-amplification

The primer and template concentrations in the PCR reaction will have to be estimated empirically in order to obtain optimal results on the gel (clear and reproducible PCR products). The normalization of the template amounts (when there is too little material for spectrophotometric measurement) in the PCR reaction is also done by testing different concentrations, and using those which yield the most similar pattern between the cDNA pools being compared. Once the template and primer concentrations have been optimized, several identical pairs of PCR reactions should be performed on the cDNAs employing different decameric primers in each one.

1. For each reaction mix on ice:
   16 µL H_2O
   2.5 µL 10× PCR-buffer
   2 µL $MgCl_2$
   1.25 µL dNTPs
   1 µL random sequence primer
   1 µL dT_{12} primer
   0.3 µL *Taq* DNA polymerase
   ~2 µL cDNA template (its concentration may have to be estimated empirically)
2. Overlay the reaction with two drops of mineral oil to prevent evaporation.
3. Run the PCR for 45 cycles of the following temperature profile: 1 min at 94°C, 1 min at 40°C, 2 min at 72°C. Extend the last cycle to 10 min at 72°C, and store the reaction at –20°C. The profile of the cycles may have to be slightly modified depending on the results obtained.

22.3.3 Resolving the PCR products

1. Prepare a 1.4 % agarose gel. The gel can already contain ethidium bromide at a concentration of 0.5 µg/mL. Alternatively stain the gel after the run.

2. Transfer 20 μL from each PCR reaction to a microfuge tube containing 3 μL of DNA 'loading buffer'.
3. Load samples of the reactions. Keep the samples of the reactions to be directly compared in adjacent lanes of the gel and run the electrophoresis.
4. Visualize the amplification products on a long wave UV transilluminator. Identify polymorphic (differentially amplified) bands and clone them in the appropriate vector, after extracting from the gel, using any standard protocol.

NOTES

1. Isolated, differentially displayed mRNA can represent false positives at a rate ranging from 10 to 70% of the cases. There are different reasons for this, ranging from cross-contamination of the initial mRNA pools at any time of their preparation (e.g. impossibility of isolating a 'pure' tissue), to problems arising from differences in the abundance of a given mRNA in different tissues. Northern analysis, whenever possible, should be the method of choice to verify the results obtained.
2. Studies using the differential display technique have shown that a large number of the bands on the gel actually consist of many co-migrating cDNA fragments. This creates some problems when trying to clone the 'correct' product. Some strategies have been developed to overcome these difficulties (Li *et al.*, 1994).
3. Although short wave UV light is better for the visualization of the PCR products, it can nick the DNA. This may cause some problems or artefacts if a reaction product is to be cloned.

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CHAPTER TWENTY THREE

Localization of RNA transcripts by in situ *hybridization*

Philip W. Ingham and Trevor Jowett

23.1 INTRODUCTION

23.1.1 Nature of technique

Techniques for the localization of mRNAs *in situ* were first developed in the late 1970s and are now widely used in many areas of biological research. They provide an extremely powerful tool for analysing patterns of gene expression, both temporally and spatially. Although other very sensitive methods are available for assaying transcript levels in different tissues and at different developmental or physiological stages (Chapters 19, 20 and 22), *in situ* hybridization is unique in its ability to reveal patterns of gene expression with cellular resolution.

The principles of the technique are very straightforward and over recent years protocols have evolved that make its practice equally simple. The objective is to fix the tissue in such a way that all the mRNA being transcribed at the time is retained within the cells transcribing the gene of interest; the mRNA is then detected *in situ* by hybridization with a probe labelled so that it can be detected either by autoradiography, chromogenic stains or fluorescence. All that is required is a supply of the appropriate stages of the organism in which the gene of interest is expressed and a cloned fragment of this gene. The procedure (shown as a flow chart in Figure 23.1) may be divided into four distinct components:

1. Preparation of tissues;
2. Preparation of probes;
3. Hybridization of probes to tissues;
4. Visualization of specific hybrids.

Tissue samples are fixed using a crosslinking fixative; the most commonly used being formaldehyde. Depending upon its size, tissue may be hybridized either intact or after sectioning; the former case has the advantage of simplicity and provides a good overall impression of the pattern of gene expression for instance in an intact embryo. However, it is often desirable to prepare sections of the material, especially for the analysis of particularly thick specimens, where probe penetration may become a problem, or for the analysis of internal organs and structures with complex

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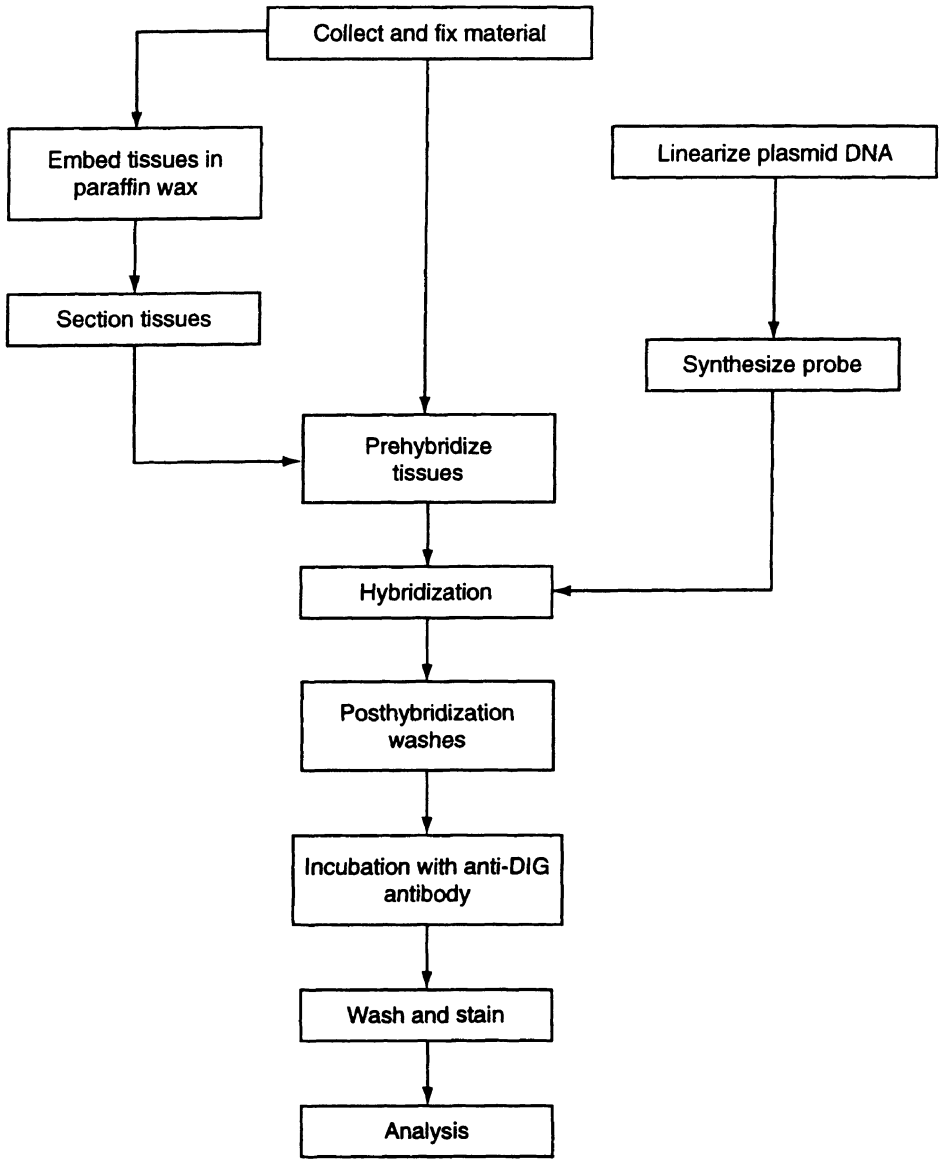


Figure 23.1 Flow chart of the steps involved in localizing RNA transcripts by *in situ* hybridization.

morphology. Although tissues can be frozen and sectioned using a cryostat, the preferred embedding medium is paraffin wax. Paraffin embedded material is easier to handle and retains superior morphology in comparison to frozen sections.

Transcripts are detected using either DNA or RNA probes. DNA probes can be prepared from cloned DNA in a number of ways, such as nick translation, random priming or polymerase chain reaction (PCR) amplification; alternatively, RNA probes may be prepared by *in vitro* transcription of the cloned DNA using the bacteriophage SP6, T3 or T7 polymerases; promoters for these polymerases are incorporated into a number of commonly used and highly versatile cloning vectors such as Bluescript™.

In general, we find the latter RNA probes to be the most sensitive and reliable and for this reason we will focus on their use.

In the earliest protocols (e.g. Akam, 1983; Hafen *et al.*, 1983; Ingham *et al.*, 1985), probes (either DNA or RNA) were labelled isotopically, usually employing ^{3}H or ^{35}S labelled nucleotides, and detected following hybridization by autoradiography. Apart from the safety considerations always associated with radioactive materials, the autoradiographic method of signal detection presents a number of other drawbacks to the use of such isotopically labelled probes. Preparation of materials for autoradiography is an expensive and time-consuming business; moreover, depending upon the abundance of the transcript to be detected, exposure times can be relatively long and must be determined empirically. The higher energy emissions of ^{35}S compared to ^{3}H significantly reduce exposure times but at the same time the longer path length decrease resolution; even with ^{3}H, the signal only approaches single cell resolution. All of these limitations are obviated by using nonisotopic labels such as biotin, digoxygenin (DIG) or fluorescein and since the publication in 1989 of a reliable method for hybridizing whole *Drosophila* embryos using DIG labelled probes (Tautz and Peifle, 1989), such labels have all but replaced their radioactive counterparts. The protocols which we describe in this chapter have been developed specifically for the use of DIG and fluoroscein labelled probes.

23.1.2 Application of the technique to the analysis of insect biology

Undoubtedly, the most spectacular application of *in situ* hybridization techniques to a problem in insect biology has been their use in the analysis of the development of the dipteran *Drosophila melanogaster.* These studies began in the early 1980s using isotopically labelled probes and sectioned material and focused on the expression of homeotic and segmentation gene expression in early embryos (reviewed in Ingham 1988; Ingham *et al.*, 1990). Nowadays, the use of DIG labelled probes on whole embryos and tissues has become a standard procedure in the analysis of any *Drosophila* gene and the technique should in principle be applicable to the analysis of gene expression in any insect species.

23.2 MATERIALS

23.2.1 Buffers and solutions

1. 10× Digoxigenin nucleotide labelling mix: 10 mM each ATP, GTP, CTP; 6.5 mM UTP; 3.5 mM digoxigenin-UTP. Store in aliquots at –20°C (available from Boehringer #1277 073).
2. 10× Transcription buffer: 400 mM Tris-HCl pH 8.0; 60 mM $MgCl_2$; 100 mM dithiothreitol; 20 mM spermidine; 100 mM NaCl; RNase inhibitor 1 U/μL. Store in aliquots at –20°C (supplied with RNA polymerase from Boehringer).
3. 10× TBE: dissolve 109 g Tris base, 55 g boric acid, 9.3 g disodium EDTA in 1L of water. pH should be 8.3. 1× TBE is 90 mM Tris, 2.5 mM EDTA, 90 mM boric acid.
4. 20×SSC for hybridization and washing: (20×SSC is 3 M NaCl, 300 mM tri-sodium citrate). Dissolve 175.3 g NaCl and 88.2 g sodium citrate in 800 mL water. Adjust the pH with 1M citric acid to 4.5 or 6.0 depending on the hybridization solution used. Adjust the volume to 1 L and sterilize by autoclaving.

5. Blocking solution for antibodies: 1× PBS, 0.1% Tween-20, 2 mg/mL BSA (BDH #44155), 5% sheep serum (Gibco BRL #035-6070H), 1% dimethylsuphoxide, DMSO (Merck-BDH #28216).
6. Carnoy's Fixative: 6 parts 100% ethanol: 3 parts chloroform: 1 part glacial acetic acid (prepare fresh on day of use).
7. Hybridization buffer

	for 10 mL	concentration
Deionized formamide	5 mL	50%
20×SSC2	2.5 mL	5×SSC
tRNA (50 mg/mL)	20 μL	100 μg/mL
Heparin (100 mg/mL)	5 μL	50 μg/mL
Tween-20	10 μL	0.1%
ddH_2O	2.45 mL	

8. NTMT: 100 mM NaCl, 100 mM Tris-HCl pH 9.5, 50 mM $MgCl_2$, 0.1% Tween-20. Make from concentrated stock solutions on day of use (the pH will decrease on storage, due to absorption of carbon dioxide).

	for 25 mL	final concentration
5 M NaCl	0.5 mL	100 mM
1 M $MgCl_2$	1.25 mL	50 mM
1 M Tris pH 9.5	2.5 mL	100 mM
Tween-20 (20%)	125 μL	0.1%
ddH_2O	20.63 mL	

9. Paraformaldehyde/PBS fixative: paraformaldehyde is dissolved in PBS at 65°C. If it does not readily dissolve add a drop or two of 1 M NaOH solution to pH 7.5. It should be cooled to 4°C and used within 2 days.
10. PBS (phosphate buffered saline): 130 mM NaCl; 7 mM $Na_2HPO_4.2H_2O$; 3 mM $NaH_2PO_4.2H_2O$. For 10× PBS mix: 75.97 g NaCl; 12.46 g $Na_2HPO_4.2H_2O$; 4.80 g $NaH_2PO_4.2H_2O$. Dissolve in less than 1 L of distilled water; adjust to pH 7.0 and to final volume of 1 L, sterilize by autoclaving.
11. PTW: PBS, 0.1% Tween-20.
12. Proteinase K (Boehringer #1000144): make up a stock solution of proteinase K at 20 mg/mL in 50% glycerol 10 mM Tris pH 7.8 and store at –20°C.
13. Ringer's solution:

	final conc.	
KCl	182 mM	13.6 g
NaCl	46 mM	2.7 g
$CaCl_2$	3 mM	0.33 g (CaCl-$2H_2O$)
Tris-HCl	10 mM	1.21 g (Tris-base)
Distilled H_2O	to 1 L	

Adjust to pH 7.2 with 1 N HCl, filter (0.47 mm) and autoclave.

14. TE: 10 mM Tris HCl, 1 mM EDTA pH 8.0. Make up with RNase-free water and autoclave.

23.2.2 Reagents

1. Anti-digoxigenin alkaline phosphatase (AP) Fab fragments (Boehringer #1093274) 150 U/200 μL.
2. BCIP, 5-bromo-4-chloro-3-indolyl-phosphate also known as X-phosphate 4-toluidine salt, (Boehringer #760994): dissolve at 50 mg/mL in dimethylformamide. Store in aliquots at –20°C.

3. DNase I, RNase free (Boehringer #776785).
4. NBT, 4-nitro blue tetrazolium chloride (Boehringer #1087479): dissolve at 75 mg/mL in 70% dimethylformamide. Store in aliquots at –20°C.
5. RNA polymerase SP6 (Boehringer #810274).
6. RNA polymerase T3 (Boehringer #1031163).
7. RNA polymerase T7 (Boehringer #881767).
8. RNase A (Pharmacia #27-0323-01).
9. RNase inhibitor (Boehringer #799017).
10. TESPA (3-aminopropyltriethoxysilane) (BDH #66023).

23.2.3 Preparing TESPA-treated microscope slides

Incubate slides overnight in acid alcohol (1% HCl in 70% ethanol). Rinse thoroughly in water. Dry in incubator. Dip in 2% TESPA in acetone for 10 s. Rinse in acetone for 10 s. Rinse in water. Drain well and bake in metal racks at 160°C.

23.3 METHOD

23.3.1 Preparation of material for hybridization

Tissue will normally contain endogenous RNases which cannot easily be eliminated, so rapid fixation is the best way of avoiding potential problems. Solutions are usually made with sterile deionized water and, if possible, autoclaved prior to use. Glass vials and microfuge tubes used for fixing and subsequent storage of embryos may be autoclaved but this is not usually necessary.

(a) Fixation

Choice of fixative is a crucial factor in determining the success of the procedure. Crosslinking agents (such as formaldehyde or glutaraldehyde) provide much better retention of nucleic acids than coagulating agents (such as ethanol/acetic acid). However, it is important not to over-fix the material as this will make the mRNA less accessible to the probe. In most cases, the fixative of choice is 4% formaldehyde.

(i) Embryos

The following protocol was developed for *Drosophila* embryos but has been successfully applied to other species such as *Musca, Chironomus* and *Tribolium* that have eggs encased in similar chorions and vitelline membranes (Sommer and Tautz, 1991; Curtis *et al.*, 1995; our own unpublished observations).

1. Collect eggs and age until the required stage. Wash eggs off the laying substrate through a coarse gauge sieve to remove as much debris as possible and collect them in a fine gauge sieve. Immerse the eggs (in the sieve) in a 7% sodium hypochlorite solution to dissolve the chorion; this takes between 2 and 5 min depending upon the number of eggs; monitor the process under the microscope. When all the chorions have disappeared, wash thoroughly in water and blot dry.

2. Using a paint brush, immediately transfer the eggs to a tube containing equal volumes of fixative solution and *n*-heptane; place tube on a roller and leave for 20 min at RT.
3. Using a pipette, remove the lower (aqueous) phase from the tube and add an equivalent volume of methanol, precooled to –70°C. Place on dry ice and agitate frequently. The embryos should burst out of their vitelline membranes and sink into the lower phase. Many protocols suggest performing this step at RT; although the yield is usually better, the morphology of the embryos, especially following sectioning, is invariably poor.
4. Remove the embryos from the bottom phase of the heptane:methanol mix, and rehydrate through a graded methanol/PBS series (7:3, 5:5, 3:7, 0:10). Any embryos that remain at the interface should be discarded.
5. Fix embryos for a further 20 min. Wash in 1× PBS, then dehydrate through 30%, 50% and 70% ethanol, 10 min each. The fixed embryos can now be stored indefinitely at –20°C prior to being hybridized (Section 23.3.3a) or sectioned (Section 23.3.1b).

(ii) Post-embryonic stages

The thick cuticle surrounding postembryonic stages presents a significant barrier to fixative solutions that necessitates a compromise in the choice of fixative. If the animal is to be fixed intact, we find that it is preferable to aim for rapid and even penetration of the tissue at the expense of optimal mRNA retention in order to obtain reasonable tissue morphology. For this purpose, the best fixative seems to be Carnoy's, but it is worth investigating others that may be more appropriate for a particular species.

Animals of the desired stage should be washed thoroughly in 1s PBS before immersing in the fixative for 1× at RT. There is no need to prick the larval cuticle, although this will aid penetration. Pupal cases should be gently broken open with fine forceps. Once fixed, the pupae are teased out of the cases. Material fixed in this way can be stored in 70% ethanol at –20°C prior to processing for sectioning (Section 23.3.1b).

As an alternative to fixing the organism intact, the tissues of interest may be dissected out and fixed separately; this has the advantage of allowing use of the optimal fixative for mRNA retention (usually 4% formaldehyde). Animals should be dissected in Ringer's solution and the desired tissues transferred to fixative. The exact time of fixation will depend upon the size of the tissue: *Drosophila* imaginal discs or brains are well fixed after 30 min at RT.

(b) Embedding fixed material for sectioning

1. Paraffin wax should be melted overnight at 58°C prior to embedding; it is essential that the wax is not overheated or melted for longer than 24 h.
2. Place the fixed material in small baskets (we make these using the caps of plastic tubes and stainless steel mesh) and dehydrate through a graded ethanol series (80%, 90%, 95%, 100%, 100%), 10 min each step for embryos and dissected tissues, 30 min each step for whole larvae or pupae. Addition of Eosin yellow to 0.1% in 95% ethanol will stain the embryos or other tissues, helping in their visualization at subsequent steps.
3. Replace the ethanol by two incubations in Histo-Clear™- (10 min for embryos, 30 min for larvae/pupae).

4. Transfer samples to a 1:1 mixture of Histo-Clear™- and paraffin wax at 58°C for 20 min (embryos) 40 min (larvae/pupae).
5. Transfer to pure wax at 58°C 2× 10 min (embryos), 20 min (larvae/pupae).

The material is now ready to embed. In order not to waste materials during the hybridization procedures, one should aim to obtain sections with as high a density of samples as possible. Orientation of small samples can be difficult and when material is not limiting, is often left to chance. Because of the shape of many insect embryos they will tend to orientate themselves with their antero-posterior axis parallel to the face of the wax block if left to settle. Thus with a little practice it is relatively easy to obtain blocks that yield a high proportion of longitudinal sections.

1. Cut the end off a Pasteur pipette and heat it briefly in a Bunsen flame. Plunge the pipette into a reservoir of molten wax for a few seconds to lower the temperature, then draw up as many embryos as possible. Then quickly, but carefully, expel the embryos into the centre of a plastic embedding mould (Figure 23.2). The mould should be placed on a hot block at 58°C so that the wax remains molten. Do not allow the drop of wax to spread to the edges of the mould as this will result in most of the embryos being lost.
2. Observe the embryos under a microscope; gentle agitation of the mould should ensure that they are spread evenly. Once the desired distribution is obtained, remove the mould from the block and quickly add more wax, being careful not to disturb the specimens. When the mould is full it can be left to harden. The embedded specimens can be stored in this form indefinitely.

If different orientations are required (e.g. to make transverse sections) it is better to use a smaller mould. Fill the mould with a drop of wax containing a high density of embryos and agitate it a little as the wax cools. This will ensure that at least some of the embryos are oriented perpendicular to the face of the block.

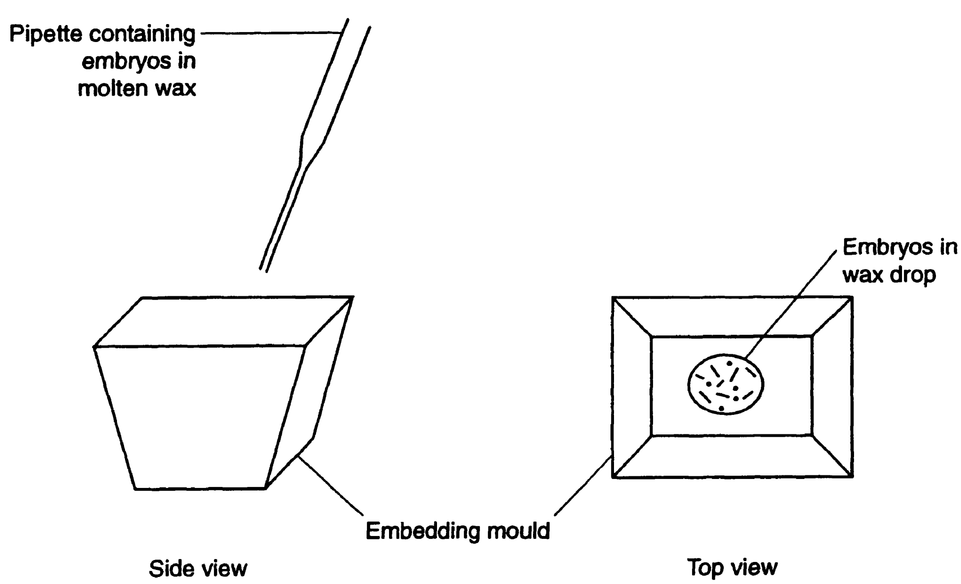


Figure 23.2 Embedding fixed embryos/tissues in paraffin wax.

To embed larger specimens, such as larvae and pupae, use a 20 × 20 mm mould. Then cover the bottom of the mould with a thin layer of molten wax and transfer the specimens using a pair of fine forceps; you must work quickly, otherwise the wax will set leaving the tissue stuck to the forceps.

(c) Cutting sections

To cut sections, you will need access to a rotary microtome. Cutting sections is a skill acquired through practice. If there is anyone within your institution who has experience of sectioning it is well worth asking them for help.

1. Remove the wax block from the mould and trim to a trapezoidal shape with a sharp razor blade (Figure 23.3). The maximum dimensions of the block should be around 5 × 5 mm.
2. Mount the block on a specimen holder using molten wax with the parallel sides of the block parallel with those of the specimen holder (Figure 23.3). Allow the wax to set for at least 20 min.
3. Lock the rotary wheel of the microtome so that the chuck does not move while you mount the blade. Take a new disposable blade and rinse in Histo-Clear™ to remove the protective oil. Keep a glass Coplin jar of Histo-Clear™ next to the microtome for this purpose. Secure the disposable blade in the blade holder and mount in the microtome.
4. Mount the specimen holder on the microtome with the smaller edge of the block uppermost and adjust the forward advance to give enough travel to cut the whole block. Slowly advance the knife towards the block and adjust the chuck so that the block face and the knife edge are parallel.
5. Begin sectioning making slow, even, downward strokes. Cut sections at 5–8 μm. Collect ribbons of sections with a paintbrush or forceps; ribbons of ~30 sections are about the longest which can easily be handled. If there is scoring or splitting of the sections, either the blade edge is damaged or there are hard particles in the wax or tissue. If the sections roll into a tight coil instead of remaining flat, the blade is blunt or dirty. Clean the blade with Histo-Clear™.

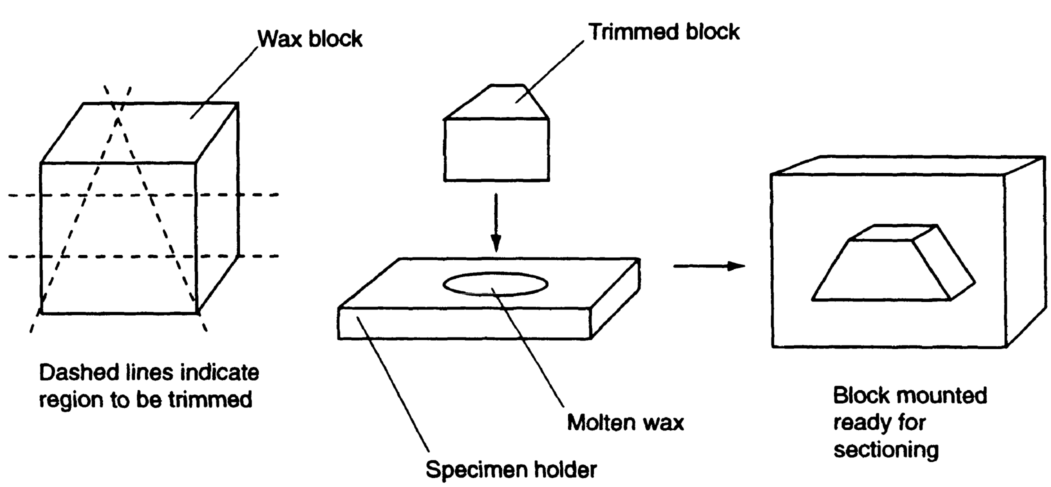


Figure 23.3 Preparation of wax blocks for sectioning.

6. Cover a TESPA coated slide (Section 23.2.3) with sterile water and layer the sections slide neatly on the surface. Allow to dry at 40°C (no hotter or else the wax will melt) for at least 4 h (can be left overnight). Alternate sections can be placed on separate slides (this is desirable, for instance, if the patterns of expression of two or more genes are to be compared) in which case the ribbons may be first laid on a clean sheet of aluminium foil and a scalpel used to separate the sections. Once dry, slides may be stored desiccated at –20°C or used immediately for hybridization.

23.3.2 Probe synthesis

Reasonable care should be taken to prevent contamination with RNases especially during synthesis of the probe. We only use diethyl pyrocarbonate (DEPC)-treated water for synthesis and storage of the probe. A single synthesis should be enough for 100–200 hybridizations so it is worth storing it in aliquots at –20°C in the presence of RNase inhibitor. It is important that the plasmid DNA is free of contaminants. Plasmid prepared by the various commercial resins is suitable (Magic™ or Wizard™ Minipreps Promega, Qiagen Plasmid Mini or Midi kit).

Linearized plasmid equivalent to 1 μg of insert DNA is digested with proteinase K (0.05 μg/μL) for 30 min at 37°C prior to phenol/chloroform extraction and ethanol precipitation. We treat with proteinase K to ensure that the DNA is free of RNase prior to riboprobe synthesis. The DNA is redissolved in TE so that 1 μg of insert DNA is in 4 μL.

1. Mix together 4 μL of linearized plasmid (1 μg of insert DNA); 2 μL of 10× Transcription buffer; 2 μL of 10× nucleotide mix (with digoxigenin-UTP or fluorescein-UTP); 20 U of RNase inhibitor. Add water to give a final reaction volume of 20 mL and 40 U of the appropriate T7, T3 or SP6 RNA polymerase.
2. Incubate the mixture for 2 h at 37°C.
3. Add 40 U of DNase I and incubate at 37°C for 15 min to remove the plasmid DNA.
4. Stop the reaction by adding 2 μL of 200 mM EDTA pH 8.0.
5. Precipitate the RNA with 2.5 μL 4 M LiCl, and 75 μL prechilled ethanol.
6. Spin down the pellet and redissolve in 100 μL of RNase-free water containing 40 U of RNase inhibitor.
7. Check the probe by running 2.5 μL on an 0.8% agarose, 1× TBE minigel. Wash the apparatus thoroughly before preparing the gel and run the samples quickly to avoid problems with RNase. If the synthesis has been successful, the probe should be visible as a single discrete band. Smears indicate premature termination of transcription and/or partial degradation – such products should be discarded.
8. The probe can be used without further treatment. It should be split into aliquots and stored at –20°C.

23.3.3 Pretreatment, hybridization and washing

Prior to hybridization, the fixed material is subjected to various pretreatments: sections are refixed which helps prevent them from falling off the slides; sections and whole tissue are then subjected to limited digestion with proteinase K. This step

makes the transcripts more accessible to the probe; although it seems to be essential for sectioned material, we find, somewhat paradoxically, that it is not necessary for whole embryos, at least for those fixed at earlier stages of development. If proteinase K digestion is performed, it is essential that the material be re-fixed immediately afterwards; failure to do so will lead to the loss of transcripts from the tissues. All solutions used are made from sterile stock solutions with deionized water.

(a) Whole tissues

All of the following steps are done in 1 mL volumes unless otherwise stated.

1. Rehydrate embryos/tissues through 70%, 50%, 30% ethanol.
2. Rinse 3× in PTW (2 min each).
3. Incubate at RT in 50 mg/mL proteinase K in PTW (a 1:400 dilution of a 20 mg/mL stock). The incubation time depends upon the tissue and the probe being used. As a guide, we find a 4 min digestion to be optimal for *Drosophila* embryos using most probes.
4. Rinse 2× 1 min in PTW.
5. Fix in 4% paraformaldehyde in PBS for 5–10 min at RT (longer fixation times seem to increase background).
6. Rinse 5× 1 min in PTW.
7. Rinse in 1:1 PTW: hybridization buffer.
8. Resuspend embryos/tissues in hybridization buffer and prehybridize for at least 1 h at 70°C; the longer the prehybridization, the better the signal to noise.
9. Remove prehybridization buffer and replace with 200–400 μL fresh buffer containing heat denatured probe (the probe should be heated to 80°C for 5 min in a small volume of hybridization buffer); the volume of probe solution used obviously depends upon the amount of RNA synthesised in the *in vitro* transcription reaction (Section 23.3.2). As a guide, try dilutions between 1:100 and 1:1000. Hybridize overnight at 70°.
10. Remove hybridization buffer containing probe and wash embryos/tissues in fresh buffer 1× 20 min at 70°C.
11. Wash in 1:1 PTW: hybridization buffer 1× 20 min at 70°C.
12. Wash in PTW 5× 20 min at RT.

The embryos/tissues are now ready for probe detection (Section 23.3.4).

(b) Tissue sections

Before prehybridization treatments, sections must first be dewaxed in an organic solvent such as Histo-Clear™. Other organic solvents, such as xylene have been reported to inhibit subsequent hybridization and/or signal detection (Tautz *et al.*, 1992) and should therefore be avoided.

1. Load slides into a glass rack and wash 2× 5 min in Histo-Clear™.
2. Rehydrate through ethanol series as follows: 2× 30 s in 100% ethanol; 1× 30 s in 95% ethanol; 1× 30 s in 90% ethanol; 1× 30 s in 70% ethanol; 1× 30 s in 50% ethanol.
3. Wash with PTW 2× 5 min each.
4. Fix with 4% paraformaldehyde in PBS for 20 min.
5. Wash with PBS, 2× 5 min.
6. Treat with 5–10 μg/mL proteinase K in PTW. The time must be determined empirically for the particular type of tissue and batch of enzyme.

7. Rinse 2× 1 min in PTW.
8. Fix with 4% paraformaldehyde in PBS for 20 min.
9. Wash 2× 5 min in PTW.
10. Quickly pass through a methanol series from 30% to 100%, then air-dry.
11. Before adding the probe, the sections may be preincubated in hybridization buffer. We do not usually find this prehybridization step to be necessary but, if included, all the prehybridization solution should be removed with filter paper before adding the probe solution to avoid dilution of the probe. Apply ~2.5 μL/cm^2 hybridization mix with probe to slides and lower a coverslip on top so that the liquid spreads over the sections. Take particular care to eliminate air bubbles. Add the hybridization solution along one side of the sections. Place the edge of the coverslip in the solution and gently lower over the remaining sections using forceps or a scalpel blade to support the coverslip. The hybridization solution should wet the sections eliminating air as the coverslip is lowered.
12. Place slides horizontally in a square plastic Petri dish containing tissue soaked in a solution of 50% formamide, 5×SSC prewarmed the box for the slides and also to the hybridization temperature. Seal the box with Nescofilm™ or Parafilm™ and incubate overnight at 65°C.
13. Transfer the slides to a Coplin jar containing 50% formamide and 2×SSC prewarmed to 65°C; there should be sufficient solution to immerse the coverslip and the coverslips should fall away. Leave for 15 min.
14. Wash for a further 30 min in 50% formamide 2×SSC at 65°C. We perform this and subsequent washes on a shaker in a hybridization oven with gentle agitation.
15. Wash twice in 25% formamide, 1×SSC, 0.5× PBS at 65°C for 30 min. This is the stringent wash.
16. Wash in PBS at RT for 5 min.

23.3.4 Immunodetection of probe

The final step in the procedure is the detection of the hybridized probe. This is achieved by incubating the hybridized material with an anti-DIG antibody coupled to an enzyme (usually alkaline phosphatase). The RNA hybrid:antibody complex is then visualized by incubation with a chromogenic substrate for the enzyme. A number of such substrates are now available for alkaline phosphatase (Section 23.4); the most commonly used is a mixture of Nitro blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP), the use of which we describe below.

(a) Whole embryos/tissues

1. Incubate hybridized specimens with anti-DIG antibody, diluted 1:2000 in PTW, for 1 h at RT.
2. Wash 4× 20 min in PTW.
3. Rinse 2× 1 min in NTMT.
4. Resuspend in 1 mL NTMT and add 4.5 μL NBT 3.5 μl BCIP. Allow reaction to proceed for up to 1 h (or more if necessary); the appearance of stain should be monitored carefully to avoid overstaining.
5. Stop reaction with several rinses in PTW.

Temporary preparations for immediate analysis can be made by mounting embryos in 70% glycerol. Use small pieces of coverslip as supports for the main coverslip to prevent the specimen being squashed. For *Drosophila* embryos, a single thickness of a No. 1 coverslip is perfect for this purpose. In order to achieve the desired orientation of the specimen it is best to mount single embryos or tissues on each slide.

If permanent preparations are required, we recommend a resin or plastic embedding medium such as Araldite or JB-4 (Polysciences). In this case the specimens must first be dehydrated. It is important to do this quickly as prolonged exposure to ethanol solutions can lead to some leaching of the stain. Fixing the stained specimens prior to dehydration helps eliminate such leaching.

1. Fix specimens in 4% paraformaldehyde in PBS for 10–20 min at RT.
2. Dehydrate briefly through 70%, 95% and 100% ethanol.
3. Remove ethanol and add 1 mL of JB-4 A+catalyst (c) solution. Allow to infiltrate until all embryos have sunk to the bottom of the tube.
4. Mix 1 mL of A+catalyst with 40 µL of JB-4 solution B. Keep cool to slow down polymerization. Remove A+catalyst from specimens and replace with the A+C+B mixture. Mix thoroughly and immediately pipette the specimens onto slides; the solution will not polymerize whilst in contact with air.
5. Arrange embryos/tissues as required and cover with a coverslip; leave to polymerize for >1 h.

(b) Tissue sections

The antibody concentrations used on sections are generally higher than those used for whole mounts.

1. Dilute antibody 1:1500 in PTW; pipette enough (around 50–100 µL) of the diluted antibody solution to cover the sections, cover with a coverslip and incubate 30–60 min at RT or overnight at 4°C in a humid chamber containing tissue soaked with PBS.
2. Wash four times for 15 min in a Coplin jar with 1× PTW at RT.
3. Wash 3× 5 min in NTMT at RT.
4. The staining reaction should be performed in the dark either by dipping the slide in a minimum volume of staining solution, or by putting a drop on the sections and covering with a coverslip. Stain until the desired signal strength is achieved (usually 45–60 min). Stop the reaction by washing with PTW.
5. For permanent mounts it is best to fix the stain by incubating in 4% paraformaldehyde in PBS for 10–20 min at RT.
6. Wash the slides in deionized water and dehydrate through an ethanol series 50%, 70%, 96%, 100% 10 min in each and then twice in Histo-Clear™ for 10 min. Add a drop of DPX mountant and a coverslip.

NOTES

Different species

The protocols described should be applicable to any insect species with minor modifications. We have seen a number of variant protocols for different insects all of which

apparently work. We therefore suspect that most of the variations are historical in origin and we recommend adhering as closely as possible to the protocols described in Section 23.3. However, it may be necessary to vary the fixation and proteinase K digestions for different embryos/tissues of different species. M.E. Akam and M. Averof (pers. comm.) report that for locust embryos, longer fixation and proteinase K treatments seem to be required (60 min in each case). In addition, various workers have found very long prehybridizations of whole tissues (up to 24 h) to be beneficial.

Problems

Poor morphology

This is usually due to poor fixation. Although PBS buffer usually gives good results, a better alternative may be PIPES. Add 1 part 37% formaldehyde solution to 9 parts PEM = 0.1 M PIPES pH 7.0, 2 mM EGTA pH 8.0, 1 mM $MgSO_4$, adjusted to pH 6.95. When removing vitelline membranes by the heptane/methanol phase partition method, it is essential that the methanol be precooled to –70°C.

No signal

The failure to detect any signal at the end of the process is an extremely frustrating, but not uncommon, occurrence. There are a number of likely causes that should be considered:

1. Probe degraded: As emphasized in Section 23.3.2, it is important that the size and integrity of the probe be checked following the synthesis reaction. Even if a probe looks good after synthesis and initially works well, it may have degraded during prolonged storage. This is particularly true if the probe has been repeatedly frozen and thawed; for this reason, it is advisable to store good probes in small (5 μL) aliquots at –70°C.
2. Probe too big: Theoretical considerations suggest that the length of the probe will influence the extent to which it is able to infiltrate tissues and thus hybridize with its target sequences. A number of protocols therefore recommend partial hydrolysis to generate shorter probe lengths, the optimum being reported to be around 250 bp. In practice, we find that probes as long as 4 kb work well with *Drosophila*. However, it may be worth hydrolysing the probe if it consistently fails to give results. This can be achieved either by boiling in hybridization buffer for at least 30 min (Tautz *et al.*, 1992) or by heating in a sodium bicarbonate buffer (Wilkinson, 1992).
3. Probe concentration: The probe dilutions that we suggest are intended only as a rough guide. In some cases, a higher concentration may be necessary; this must be determined empirically.
4. Probe complexity: The complexity of the probe, i.e. the amount of the transcribed sequence that it includes rather than its absolute length, along with the abundance of the transcript to be detected, will also influence the signal level. If genes are expressed at very high levels, then a fragment comprising as little as 100 bp of the transcribed sequence will be sufficient to use as a probe. However, wherever possible, probes should be derived from fragments containing at least 500 bp of the total transcribed sequence.
5. Poor preservation of RNA: Good fixation of the starting material is essential for efficient retention of RNA. If the material is not fixed adequately, a lot of the

transcript will be lost. Fixation of *Drosophila* embryos in ethanol:acetic acid, for instance leads to a 10× reduction in signal level compared to equivalent embryos fixed in formaldehyde. Similarly, longer fixation in formaldehyde can increase signal levels (but see below). RNA can also be lost following the proteinase K digestion so it is important always to re-fix the material after this step.

6. Accessibility of transcript: Whilst fixation is essential for preservation of transcripts, over-fixation can result in their being inaccessible to the probe. Strong crosslinking reagents, such as glutaraldehyde, should be avoided if possible. If they are used, longer digestion with proteinase K will be required. Even after formaldehyde fixation, proteinase K digestion leads to a significant increase in signal levels.
7. Hybridization conditions too stringent: The conditions that we suggest for the hybridization are extremely stringent (in fact well above the predicted melting temperatures of RNA:RNA hybrids!) – the hybridization temperature may therefore be reduced, to as low as 50°C to increase the level of hybridization. However, one should be aware of the possibility of cross-hybridization with related sequences as the stringency is lowered.
8. Dead antibody: Antibodies can become inactive apparently overnight, especially if stored under inappropriate conditions. For prolonged life, it is best to aliquot the antibody and store at 4°C.
9. Staining buffer pH: A trivial reason for the staining reaction not working is the use of the wrong buffer, or a change in pH of the correct buffer. If you see no colour after prolonged incubation in the alkaline phosphatase substrates, check that you have used the NTMT buffer and not PTW. If you have not, the experiment can be rescued simply by washing in NTMT a few times and then repeating the staining reaction. If you have used the correct buffer but no stain appears, check that the pH is 9.5. It is best always to make the NTMT just prior to use from concentrated stock solutions.

High background staining

1. Probe concentration too high: This should be determined empirically.
2. Nonspecific binding of antibody: Many protocols recommend that the anti-DIG antibody be preabsorbed against fixed material before use in probe detection. In practice, we find that such preabsorption makes little or no difference, at least with *Drosophila* material; however, if persistently high background staining is experienced, it is worth including this step: incubate the anti-DIG antibody for at least 4 h at RT (or overnight at 4°) at a 1:400 dilution in PTW + 2% sheep serum (to stabilize the antibody), 1% azide, with a large volume of fixed embryos. Use about 50 μL of embryos in 1 mL. The diluted antibody is stable in this solution for several months so this step can be done in advance of its use.
   Nonspecific binding can also be reduced by blocking the hybridized tissues, prior to incubation with the antibody: Incubate the hybridized embryos/tissues in 1% Boehringer Blocking reagent in 0.1 M Malic acid, 0.15 M NaCl, for at least 1 h at RT.
3. Endogenous alkaline phosphatase activity: Some tissues have high levels of endogenous enzyme activity; this can be specifically inhibited by addition of Levamisole to the staining buffer, to a final concentration of 5 mM.
4. Other causes: Prehybridization of tissues, especially whole embryos seems to reduce background staining significantly. The rule seems to be the longer the

better. Some protocols report that rinsing embryos in 50% ethanol 50% xylene immediately after removal of the vitelline membrane followed by soaking in 100% xylene for several hours and further washes in 50% ethanol 50% xylene significantly reduces background.

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CHAPTER TWENTY FOUR

In situ localization of proteins in whole mounted tissue

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24.1 INTRODUCTION

Over the last decade of biomedical research, the development of techniques that allow one to visualize gene expression patterns and the tissue-specific distribution of gene products has become a central issue. The recent progress in determining the distribution of specific transcripts using *in situ* hybridization techniques involving DNA or RNA probes was almost exclusively dependent on recombinant DNA techniques (Chapter 23). Similarly, antibody staining techniques which allow one to visualize the tissue- and cell-specific distribution and compartmentalization of proteins had been dependent on the availability and purity of antigens and on their success in challenging the immune response of injected animals serving as a biological test tube for generating the desired antibodies. Once antibodies could be purified, they were used as a tool on the one hand to demonstrate the presence of their antigen for the relatively rapid purification of the antigens by affinity chromatography or immunoprecipitation, and on the other hand to visualize *in situ* the antigens in fixed tissue by virtue of 'antibody staining'. For the latter application, antibodies are labelled by various means including isotopes, fluorescent tags or enzymatic activities to generate visible reaction products at the site of the antigen.

Recent progress has been made with respect to the production of specific antibodies and their detection. With recombinant DNA techniques, proteins containing nonspecific histidine tags (i.e. six to eight histidines) at either the N- or the C-terminus can be generated. This tag allows purification of the tagged protein in large quantities by using a simple one-step affinity purification protocol (Arnold, 1991; Hemdan *et al.*, 1989). In addition, commercially available enzyme or dye-labelled secondary antibodies can then be used which target antigens on the primary antibodies; these are specific for the animal in which the primary antibody was produced (usually horse, goat, rabbit, rat, mouse or chicken). These secondary antibodies allow visualization of the antigen/primary antibody-complex without going through an elaborate labelling procedure for each individual primary antibody. Furthermore, the labelling of the secondary antibody enhances the signal-to-noise ratio of the antibody staining, and it is therefore more sensitive than staining procedures involving labelled primary antibodies.

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Here we describe procedures which allow visualization of specific proteins *in situ*. The staining methods are described for whole mounted embryos and imaginal discs of *Drosophila*. These tissues serve as examples for the application of the techniques described, but they can be adapted with different degrees of modification both to other kinds of insect tissue and to other arthropods (Patel *et al.*, 1989; 1992; 1994; Schmidt-Ott *et al.*, 1994; Curtis *et al.*, 1995).

24.2 MATERIALS

24.2.1 Materials for egg collection and handling of embryos

1. 'Fly cage': Using a fine, flame-heated needle, make several holes (smaller than flies) into the bottom of a large, cylindrical plastic vial (Greiner GmBH, Stuttgart; catalogue #226170).
2. Agar plates serving as surface for egg-laying: Autoclave an agar solution (85 g agar in 3 L water). Let it cool to 60°C while stirring. Add 1 L of commercial apple juice supplemented with 100 g sugar (heated to 60°C), 40 mL ethanol and 6 g Nipagin. Stir well and pour the mixture into Petri dishes which are slightly larger than the diameter of the fly cage. Store agar plates at 4°C until used.
3. Fresh yeast: Using commercial baker's yeast, prepare a paste by adding a small amount of water to the yeast in a 100 mL beaker. Transfer the paste with a spatula to a 50 mL syringe. Close the syringe with its needle device, removing the needle itself and leaving the plastic part. Apply the yeast to agar plates using the syringe. Store at 4°C for as long as 1 week.
4. 'Mesh cup' for egg handling: Remove the lid of a 1.5 mL microfuge tube. Cut the upper part of the tube (about 5 mm from the top) with a flame-heated blade. Press the resulting ring on a piece of metal mesh (of course, mesh size needs to be smaller than embryos) heated on a heating plate so that the plastic surface melts and fuses to the mesh. Remove the excess mesh which extends over the edges of the plastic ring such that the mesh matches the size of the ring.

24.2.2 Reagents for fixing and staining of embryos

1. Distilled water.
2. 50% bleach solution: 50 mL distilled water, 50 mL commercial bleach. Store at RT.
3. Fixing buffer: Dissolve 46 g HEPES sodium salt (100 mM) in approximately 950 mL distilled water, add 4 mL $MgSO_4$ (1 M stock solution, 2 mM final concentration) and 4 mL EGTA (0.5 M stock solution, 1 mM final concentration). Adjust to pH 6.9 and 2 L final volume. Autoclave. Store at 4°C.
4. 37% Formaldehyde solution, heptane and methanol.
5. 10×BBS: Dissolve 12.1 g Tris (100 mM), 32.1 g NaCl (550 mM), 29.8 g KCl (400 mM), 14.2 g $MgCl_2 \cdot 6H_2O$ (70 mM), 7.4 g $CaCl_2 \cdot 2H_2O$ (50 mM), 36 g glucose (200 mM) and 171 g sucrose (500 mM) in distilled water (final volume: 1 L). Adjust pH to 6.95. Make aliquots of 45 mL in 50 mL Falcon tubes. Store at –20°C.
6. BBT: This solution contains 45 mL 10xBBS, 450 mg bovine serum albumin (fraction V; Merck; catalogue #12018) and 4.5 mL Tween 20 (10% stock solution),

adding distilled water up to a final volume of 450 mL. Store at –20°C in 50 mL aliquots in Falcon tubes.

7. Primary antibodies: In addition to primary antibodies which can be obtained from the producing labs directly, the number of commercial antibodies is increasing. For example, a number of *Drosophila* primary antibodies can now be found in the Developmental Studies Hybridoma Bank (University of Iowa, 436 Biology building, Iowa City, IA 52242, USA). To get optimal results, test several dilutions of the primary antibody over a range of one order of magnitude around the concentration value recommended for use by the manufacturer.
8. Secondary antibodies: Several companies supply a variety of differently tagged secondary antibodies against sera produced in a number of animals. In our experience, amongst others, the following antibodies worked quite well: biotinylated horse anti-mouse IgG (Vector laboratories; catalogue #BA2000), biotinylated goat anti-rabbit IgG (Vector laboratories; catalogue #BA1000), alkaline phosphatase-conjugated goat anti-mouse IgG (Jackson Immunoresearch; catalogue #115-056-062), alkaline phosphatase-conjugated goat anti-rabbit IgG (Jackson Immunoresearch; catalogue #111-056-045), fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Jackson Immunoresearch; catalogue #111-095-003) and FITC-conjugated anti-rat IgG (Jackson Immunoresearch; catalogue #112-095-003) and Cy3™-conjugated goat anti-rabbit IgG (Jackson Immunoresearch; catalogue #111-166-003). Of course many other antibodies are available from these and other companies.
9. Normal goat serum (Vector laboratories; catalogue #S-1000).
10. 10×PBS: Dissolve 75.97 g NaCl (1.3 M), 12.46 g $Na_2HPO_4{\cdot}2H_2O$ (70 mM), 4.68 g $NaH_2PO_4.H_2O$ (30 mM) in distilled water to give a final volume of 1 L; adjust to pH 7.0. Store at RT.
11. PBT: Mix 100 mL of 10×PBS, 10 mL of Tween 20 (10% stock solution; final concentration 0.1%) and 890 mL of distilled water.
12. Vectastain Elite ABC Kit (Vector laboratories; catalogue #PK6100). Store at 4°C.
13. Staining dish: Any small glass container where the enzymatic reaction can be developed.
14. DAB solution (10 mg/mL): Dissolve 50 mg diaminobenzydine (DAB, Sigma) in 5 mL of 50 mM Tris pH 7.5. Store in aliquots of 500 µL at –20°C. *DAB is hazardous, therefore, handle with gloves and discard as toxic waste.*
15. H_2O_2 (30%.): Store at 4°C.
16. Siliconized staining dish: Add 200 µL of Repel silane (Pharmacia Biotech, Uppsala. Catalogue #17-1331-01) to a staining dish so that the silane solution wets the inner surface of the dish. Let the solution evaporate in a fume hood, then wash the dish with water and afterwards with 100% ethanol.
17. Alkaline phosphatase buffer (APB): Dissolve 6 mL NaCl (5 M stock solution; final concentration 100 mM), 15 mL $MgCl_2$ (1 M stock solution; final concentration 50 mM), 30 mL Tris-HCl (pH 9.5, 1 M stock solution; final concentration 100 mM) and 3 mL Tween 20 (10% stock solution; final concentration 0.1%) in distilled water to give a final volume of 300 mL. Store at RT.
18. Nitroblue tetrazolium chloride (NBT; Boehringer Mannheim; catalogue #1383213). Store at –20°C.
19. X-phosphate (X-Pho; Boehringer Mannheim; Catalogue #1383221). Store at –20°C.

24.2.3 Reagents for embedding stained embryos

1. Canada Balsam for microscopy (Serva; catalogue # 26896).
2. Mowiol/4-88 embedding medium: Stir 12 g glycerol (analytical grade) and 4.8 g Mowiol/4-88 (Hoechst) in a 100 mL beaker (1 h at RT). Add 12 mL distilled water and stir (2 h at RT). Add 24 mL 0.2 M Tris pH 8.5 and stir for 2–3 h at 50°C. Centrifuge in 50 mL Falcon tubes (15 min at 6500 rpm). Aliquot in small glass tubes and keep at –20°C.
3. Araldite embedding medium: Stir 39 g of Araldite (Araldite CY 212; SERVA; catalogue #13824) and 36 g of Araldite hardener (HY964; SERVA; catalogue #13826) in a 200 mL glass beaker. Mix as slowly as possible with a magnetic stirring device for 2 h. Then add dropwise 1.5 mL of accelerator solution (Araldite accelerator DY964; SERVA; catalogue #13825) while stirring for 20 min. Distribute the viscous solution into 4 Falcon tubes (50 mL). Centrifuge (10 min at 5000 rpm) to remove the air bubbles. Store at –20°C. Since the Araldite solution is very viscous, handle it with pipettes containing a large opening, i.e cut a 1 mL pipetteman tip about 1 cm from the bottom. *Note: Araldite is hazardous! Handle with gloves and discard as toxic waste.*
4. Xylene/Araldite solution: Mix 0.5 mL of xylene and 0.5 mL of Araldite in a microfuge tube; prepare fresh solution each time.
5. Capillaries for embedding: Use Hilgenberg capillaries (Hilgenberg GmbH; catalogue #1421602). These glass tubes are 65 mm long with an outer diameter of 0.25 mm and a wall width of 0.025 mm.
6. PBX2: Mix 100 mL of 10xPBS, 20 mL of Triton X100 (10%) and 880 mL of distilled water.
7. Forceps: for tissue dissection, use forceps such as Dupont no.5.
8. Other chemicals: Dimethyl sulfoxide, DePeX (Serva; catalogue #18243), ethanol (100% and 70%) and xylene.

24.3 METHODS

The following methods are designed to visualize the *in situ* distribution of specific proteins in fixed, whole mounted *Drosophila* embryos and in fixed imaginal discs, respectively. The applications are not restricted to these examples. However, the procedures involved may need to be modified in order to be successfully adapted to materials of other sources.

24.3.1 Embryo collection

1. Up to 300 flies (3–5 days after eclosure) are anaesthetized with either CO_2 or ether vapour. Transfer the flies to a fly cage sitting on top of an agar plate containing a small amount of freshly prepared yeast paste in its centre. Allow flies to deposit the eggs while keeping the cage at 25°C in a humid and undisturbed environment. The first overnight collection yields a high number of unfertilized eggs, particularly when the females have been kept in isolation for a period of time. In order to obtain the full spectrum of embryonic stages, flies are kept under the above described conditions for 18 h overnight. Make sure that the

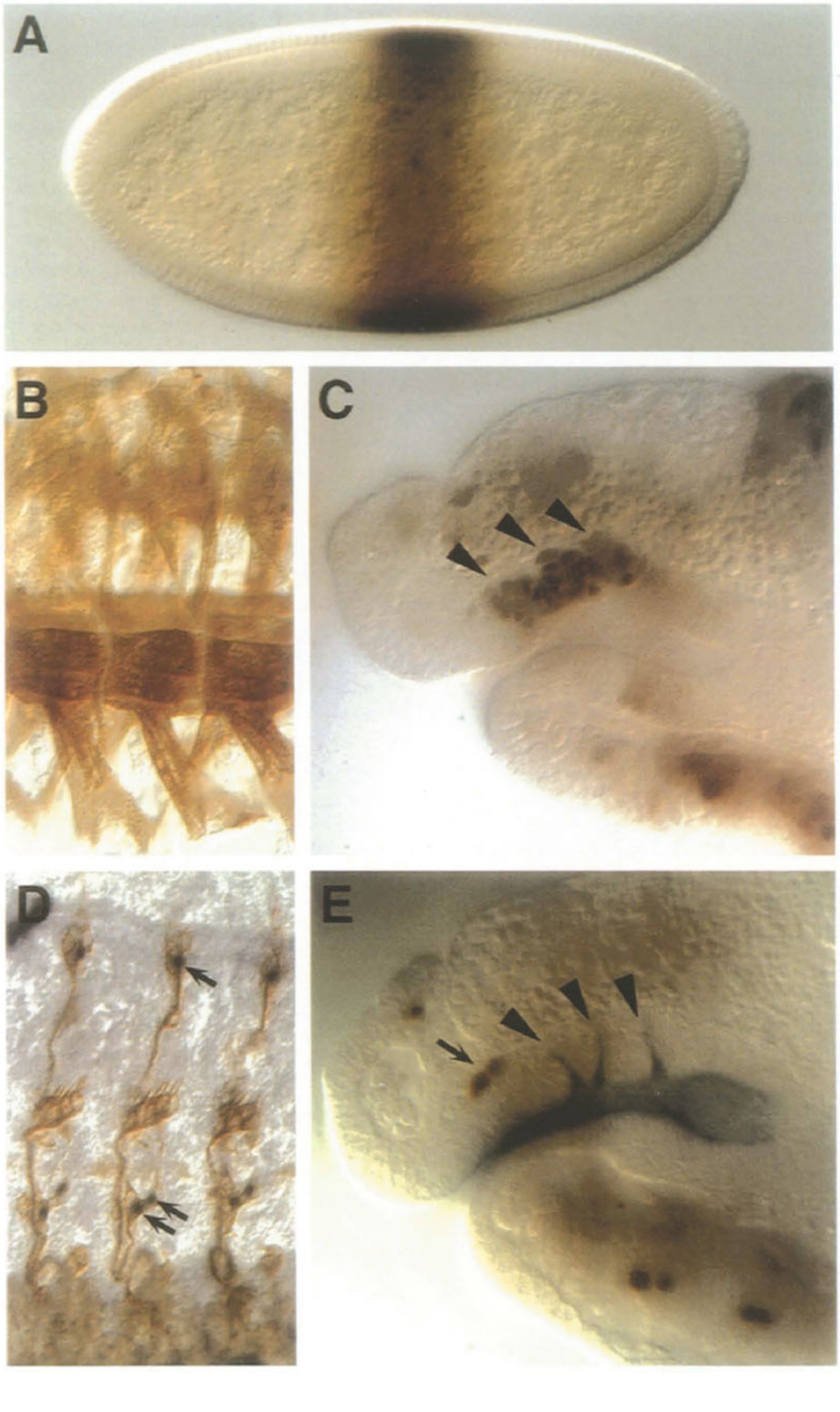

A
B
C
D
E

Plate 1 Antibody staining patterns obtained with the procedures described. (A) Anti-Krüppel antibody staining showing that the *Krüppel* protein is expressed in a restricted region of the cellular blastoderm embryo (Gaul and Jäckle, 1987); anterior is left, dorsal side up. Primary antibody: polyclonal rabbit anti-Krüppel-antibodies (gift of Pilar Carrera); secondary antibody: biotinylated goat anti-rabbit IgG antibodies; peroxidase reaction. (B) Anti-myosin heavy chain-antibody staining showing cytoplasmic localization of the protein in muscles (enlarged lateral region of an embryo at a late stage of development). Primary antibody: polyclonal rabbit anti-myosin heavy chain-antibodies (gift of Dan Kiehart); secondary antibody: biotinylated goat anti-rabbit IgG antibodies; peroxidase reaction. (C) Head region of an embryo at mid-stage of embryogenesis showing nuclear localization of the *Krüppel* protein in the anlage of the stomatogastric nervous system (González-Gaitán and Jäckle, 1995). Note three invaginations (arrowheads). Staining and antibodies were as in (A); the picture was taken from an embryo embedded in a capillary. (D) Double-stained embryo showing in brown (peroxidase reaction) the peripheral nervous system as revealed by the neuron-specific antibody Mab22C10 (Fujita *et al.*, 1982) and in blue (arrows) the nuclear localization of the protein encoded by the gene *forkhead* (Weigel *et al.*, 1989). Note the blue staining in specific neurons and brown staining associated with membranes that make it possible to see the cell body and the axons of the neurons. Primary antibodies: polyclonal guinea pig anti-*forkhead*-antibodies (gift of Pilar Carrera) and monoclonal mouse Mab22C10; secondary antibodies: Alkaline phosphatase-conjugated goat anti-guinea pig IgG-antibodies and biotinylated horse anti-mouse IgG; peroxidase and phosphatase reactions. (E) Double-stained embryo showing nuclear localization of the **reversed polarity (repo)** protein (Halter *et al.*, 1995) in glial cells (brown; arrow) and localization in the apical membrane of ectodermal epithelial cells of the **crumbs** protein (Tepass and Knust, 1993) (blue). Note the three invaginations of the stomatogastric nervous system (arrowheads; compare with C). Primary antibodies: polyclonal rabbit anti-**repo**-antibodies (gift of Joachim Urban) and monoclonal mouse anti-**crumbs** (gift of Elisabeth Knust); secondary antibodies: biotinylated goat anti-rabbit IgG antibodies and alkaline phosphatase-conjugated goat anti-mouse IgG-antibodies; peroxidase and phosphatase reactions.

agar plates are exchanged at least every 24 h to avoid old embryos whose cuticle has been already secreted and larvae; these stages will not stain because their cuticle impairs the permeability that is necessary for the antibodies to enter the tissue.

2. Using a small painting brush, transfer the embryos from the agar plate to a 'mesh cup' which is partially immersed in a dish with water. Wash the embryos thoroughly by running distilled water through the cup. Finally remove the excess liquid by blotting the mesh cup on a piece of Whatman paper.

24.3.2 Fixation of embryos

1. Dechorionate the embryos by immersing the mesh cup in the 50% bleach solution for 3.5 min. Remove excess bleach by blotting on Whatman paper and rinse the embryos with distilled water. Remove water by blotting on Whatman paper.
2. Prepare fresh fixing solution in a 20 mL glass scintillation vial by mixing 5 mL heptane, 4.5 mL fix buffer and 0.5 mL of 37% formaldehyde solution. Note that a heptane/aqueous interface appears. Transfer the embryos from the mesh cup to the scintillation vial: take the embryo-containing mesh cup with forceps, immerse and shake it in the upper heptane phase until most (or better all) embryos have left the mesh cup. Remove the mesh cup and close the scintillation vial tightly. Shake the vial vigorously for 20 min at RT using a shaking device. Wait until the two phases have separated and embryos accumulate in the heptane/aqueous interface. Remove the lower aqueous phase with a Pasteur pipette as completely as possible without taking the interface.
3. Add 10 mL methanol and shake vigorously (by hand or better by vortexing) for 1 min. Let the methanol/heptane phases separate. Embryos which have lost their vitelline membrane will sink from the interface into the methanol. With a Pasteur pipette remove the upper heptane phase including the interface (which contains the embryos still in their vitelline membrane) and most of the methanol. Add 10 mL of fresh methanol, shake briefly and let the embryos sink again to the bottom of the vial. Collect the embryos on one side of the vial (by turning and flicking the vial). Transfer them to a microfuge tube by pipetting (Eppendorf, Gilson or equivalent pipetteman, using 1 mL pipette tips from which the bottom part is cut to enlarge the opening so that the embryos are not damaged). Remove the remaining methanol and replace it by adding 1 mL of fresh methanol. Gently shake for 5 min, let the embryos sink to the bottom and replace the methanol by 1 mL of fresh methanol. At this stage, the embryos can be stored at –20°C in a freezer for a period of time that depends on the stability of the antigen, but ranges from a couple of weeks to several months.

24.3.3 Staining of embryos by labelled, secondary antibodies

We refer to antibodies directed against the antigen of choice as 'primary antibodies' (1°Ab). If they are coupled to a detectable tag, such as isotopes, fluorochromes or enzymes, they can be directly used to localize the antigen when forming the antigen–antibody complex *in situ*. We describe here instead procedures involving 'secondary antibody' (2°Ab) against nontagged primary antibodies, which yields an

enhanced signal-to-noise ratio relative to directly labelled primary antibodies. Furthermore, labelled secondary antibodies directed against immunoglobulins of many species are commercially available and convenient to use.

The principle of this procedure is based on the detection of antigen–antibody complexes (involving primary antibodies) by secondary antibodies directed against the IgG fraction of the primary antibody-producing animal species. The secondary antibodies can be coupled to fluorochromes (which can be visualized by excitation at a given wavelength), or conjugated either with an enzyme (alkaline phosphatase or peroxidase) or biotin (see below). The peroxidase can be visualized by a brown reaction product generated from its H_2O_2 and DAB substrates and the alkaline phosphatase, by a dark blue reaction product generated from its X-phosphate substrate. Other enzyme tags, such as β-galactosidase or glucose oxidase, are also commercially available.

The use of biotinylated secondary antibodies is recommended when the amount of antigen is limiting and the staining reaction needs to be enhanced, but serves well for general application. The enhancement occurs through avidin, which contains four highly specific binding sites for biotin. If avidin is added to a biotin-conjugated enzyme, such as peroxidase, each molecule of avidin can agglutinate up to three molecules of the enzyme, leaving one binding site free (-Avidin-3×Biotin-Enzyme). The triple antigen/primary antibody/secondary antibody complex is labelled by one or several biotin molecules, which are coupled to the secondary antibody (Antigen/1°Ab/2°Ab-Biotin). Then, by exposing this complex to the avidin/biotin/peroxidase complex, at least three instead of one enzyme tag can be provided to the antigen (Antigen/1°Ab/2°Ab-Biotin-Avidin-3xBiotin-enzyme), which results in a considerable enhancement of the signal-to-noise ratio.

Independent of the kind of antibody label, there is always a problem with background staining due to the binding of antibodies in the serum (both for the primary and the secondary antibody) to antigens other than the one of interest in the sample. One way to reduce such nonspecific effects and to obtain optimal stainings is to 'preadsorb' the primary and secondary antibody, i.e. to subtract the nonspecific antibodies by incubating the serum with large amounts of fixed tissue of the same origin as the one to be examined.

(a) Preadsorption of antibodies

Dilute the antibody solution 1:10 in BBT by adding 50 μL of the antibody solution and 450 μL BBT to 250 μL of fixed and rehydrated embryos (see below) in a 1.5 mL microfuge tube. Gently shake this solution (in a device such as a rotator) between 10 h and one day at 4–7°C. Remove the antibody solution which is now ready for use. It can be stored at 4–7°C for several months.

(b) Staining of embryos

1. Rehydration of embryos: Between 10–100 μL of fixed embryos (Section 24.3.2) are transferred to a microfuge tube. Always transfer embryos with a cut pipette tip to avoid damaging them. Methanol is carefully removed. For rehydration, embryos are washed 3× with 1 mL BBT for 20 min each. Washes and incubations are done by gently shaking the microfuge tubes in the rotator at RT.
2. Incubate rehydrated embryos with 300 μL of the preadsorbed primary antibody (directed against the fly antigen) at the appropriate dilution in BBT (given by the

producer or determined empirically). Shake the microfuge tube gently at 4–7°C overnight. All subsequent steps are performed at RT.

3. Remove antibody solution and wash the embryos by incubation in 1 mL BBT for 20 min.
4. Dilute 45 μL goat serum in 1.5 mL BBT. Wash embryos twice with 0.5 mL BBT/goat serum for 20 min. This step is necessary to reduce the nonspecific binding of the secondary antibody.
5. Remove the supernatant and add the preadsorbed secondary antibody (for example: biotinylated goat anti-rabbit IgG antibody if the primary antibody was produced in rabbit). This antibody is diluted 1:500 by mixing 10 μL of the preadsorbed secondary antibody (already diluted 1:10 in BBT for preadsorption as described above) in the remaining 500 μL of BBT/goat serum. Incubate for 2 h at RT (or at 4°C overnight) while gently shaking the microfuge tube.
6. Prepare the avidin/biotin-coupled peroxidase complex: Mix 0.5 mL PBT, 10 μL avidin (solution A) and 10 μL biotin-coupled peroxidase (solution B) of the Elite ABC kit. Incubate the solution for 30–40 min at RT before adding to the embryos. A similar kit exists where biotin is coupled to alkaline phosphatase (or other enzyme tags), but in single stainings better results are obtained with peroxidase as the enzyme tag.
7. Wash the antibody-treated embryos 4× for 10 min in 1 mL PBT, add the AB mix and gently shake the embryo-containing microfuge tube for 30 min. Wash 4× for 10 min with 1 mL PBT.
8. Replace the last wash solution with 0.5 mL PBT to which 25 μL of 10 mg/mL DAB is added. Incubate for 5 min.
9. Transfer the content of the microfuge tube to a siliconized staining dish using a pipette.
10. Add 5 μL of a freshly prepared 0.3% H_2O_2. Swirl the staining dish so that embryos move gently in the solution and the H_2O_2 mixes with the DAB solution. To intensify the signal one may add 4% $NiCl_2$ to this solution which results in turning the brown peroxidase precipitate into a black one. In order to monitor the reaction, the development of the brown precipitate is observed using a dissecting microscope. Once the desired staining intensity is reached, stop the peroxidase reaction by removing the substrate with five rapid washes with excess PBT.
11. Transfer embryos back into a microfuge tube. To store or embed (see below) replace the PBT with 70% ethanol. Stained embryos can be stored in 70% ethanol at 4°C at least for several weeks without losing their staining intensity.

Examples of stainings performed with this protocol are shown in Plate 1 A–C.

If fluorochrome-conjugated secondary antibodies instead of biotin-tagged secondary antibodies are used, the following adjustments to the protocol should be made:

1. Rehydrate the embryos as described.
2. Incubate embryos with primary antibodies as described.
   Remove antibody solution and wash embryos as described.
3. Incubate in a solution containing secondary, fluorochrome-coupled antibodies (either FITC- or Cy3-coupled) directed against the primary antibodies (as otherwise described above). Cy3™ is a fluorochrome that behaves like rhodamine. Keep in the dark to avoid bleaching of the fluorochrome.
4. Wash 4× for 10 min in 1 mL PBT.

5. To embed incubate first 10 min in 50% Mowiol/4-88 in PBS and then 10 min in pure Mowiol. Mowiol/4-88 impairs the bleaching of the fluorescence with time. Therefore you can keep the preparation in the dark at 4°C as well as expose the preparation to the excitation light for a longer period of time. Transfer embryos to a microscope slide, cover with a coverslip and seal with nail polish. Visualize under the microscope using a mercurium lamp with either a '515' filter (FITC) or '550' (Cy3) filter setting.

24.3.4 Double staining for the detection of two antigens simultaneously

Due to the ability of producing primary antibodies in different animal species, differentially labelled secondary antibodies can be used to discriminate between different antigens to which the primary antibodies are bound. This makes it possible to visualize simultaneously the sites of two different antigens within the same specimen, provided that the secondary antibodies are tagged by different labels. For such 'double-label antibody staining reactions', the protocol needs to be modified:

1. Fix and rehydrate the embryos as described in Section 24.3.3.
2. Incubate embryos with a mixture of two preadsorbed primary antibodies which were produced against different antigens in two different animal species. Incubate overnight at 4°C.
3. Wash for 20 min with 1 mL BBT at RT shaking gently.
4. Prepare a BBT-goat serum solution with twice the normal amount of goat serum (90 μL goat serum in 1.5 mL BBT). Wash embryos 2× for 20 min using 0.5 mL of this solution each time.
5. Remove the supernatant and add a mixture of secondary antibodies (for example biotin-conjugated and alkaline phosphatase-conjugated antibodies) in the BBT/goat serum solution.

6–10. The staining procedure continues as for the single staining protocol described in Section 24.3.3.

11. Once the peroxidase staining is finished, rinse with 1 mL APB, i.e. add, let embryos settle and remove.
12. Wash 3× 10 min in APB by gently shaking the microfuge tube.
13. Prepare fresh alkaline phosphatase staining solution by adding 4.5 μL NBT to 0.5 mL APB; mix well. Incubate embryos in this solution for 5 min and subsequently transfer them to a staining dish. Add 4.5 μL X-phosphate to the staining solution and mix well. Monitor the development of a purple precipitate under a dissecting microscope. Stop the enzymatic reaction by changing the pH back to 7, washing the embryos several (4–5) times in excess PBT.
14. Transfer embryos back into a microfuge tube.
15. Exchange PBT with 70% ethanol. Store overnight at 4°C; this turns the initial purple reaction product into a dark blue.

Examples of stainings performed with this double staining protocol are shown in Plate 1 D and E.

For simultaneous detection of two antigens in the same cells, usage of fluorescence combined with confocal microscopy instead of histochemistry is recommended. The protocol is essentially the same as described, namely the two primary antibodies are

mixed, the two secondary antibodies are also mixed and double the amount of serum is added to block. After incubation with the secondary antibodies, embryos are washed 4× with PBT and then embedded in Mowiol as described previously.

24.3.5 Examination of stained embryos

(a) Embedding procedure using a coverslip for microscopic analysis

1. Wash embryos in 70% ethanol rocking the microfuge tube for 10 min.
2. Wash 2× 10 min in 100% ethanol.
3. Transfer embryos to the slide with as little ethanol as possible by letting the embryos sink in a cut blue tip and dripping them onto the slide.
4. Remove most of the ethanol and add Canada Balsam. Mix using a needle to distribute the specimens. Wait 1 h before adding the coverslip. Analyse with Nomarski optics.

An example of a stained embryo embedded according to this protocol is shown in Plate 1A.

(b) Embedding in capillaries

Embedding of the embryos in capillaries allows for rotation of the specimen during microscopic analysis, which means the embryo can then be viewed from different angles. This is particularly important when observing highly three-dimensional structures, such as the larval gut, the brain, or the stomatogastric nervous system. This is a procedure modified from a protocol from Technau's lab (Prokop and Technau, 1993).

1. Wash for 10 min in 70% ethanol, rocking the microfuge tube.
2. Remove as much 70% ethanol as possible, and rinse with 1 mL 100% ethanol.
3. Wash 2× for 10 min using 1 mL 100% ethanol.
4. Wash for 10 min with 1 mL 1:1 100% ethanol/xylene (premix before adding to embryos).
5. Shake gently for 10 min in 1 mL xylene.
6. Remove the xylene and add 250 μL of a mixture of 1:1 xylene/Araldite (premix before adding to embryos). Incubate for 15 min in the xylene/Araldite solution and transfer with the embryos to a nonsiliconized glass staining dish.
7. Centre the embryos in the dish, remove as much of the xylene/Araldite solution as possible (with a narrow, white tip of a 10 μL pipetteman which is mounted on top of a blue tip). Add 300 μL Araldite. Thoroughly, but gently, mix the embryos with the Araldite (use a dissection needle). Incubate them for at least 1 h and up to 3 h. At this point, embryos can be stored at –20°C if the staining dish is covered with Parafilm. When embryos are to be embedded after storage, let the dish warm up to RT before removing the parafilm to avoid water condensation in the Araldite.
8. Allow 2–3 mm of Araldite to enter a capillary by capillary action.
9. Align the embryo and capillary such that one end of the embryo is very close to the opening of the capillary so that it moves into the capillary by capillary force. Do not move the capillary until the embryo is completely inside, otherwise it may come back out or be tilted inside the capillary.

10. Embed several embryos into one capillary leaving a little space between them. Normally up to 10 embryos can be sucked into each capillary, with the last ones entering the capillary very slowly.
11. Allow some Araldite to enter after the last embryo. Remove Araldite outside the capillary by an acetone soaked Kimwipe, and seal the end of the capillary with plasticine (modelling clay). Wipe again to take off extra plasticine.
12. Take a commercial tape to make a 'flag' on the unsealed end of the capillary. By using a needle you will then be able to rotate the capillary in its 'holder' during microscopic inspection by pushing on the sides of the flag.
13. Store the capillaries inside 'garages': wider capillaries (regular capillaries for embryo transformation and transplantation: Hilgenberg catalogue #1413017. 1 mm outer diameter, 10 cm long) cut in 5 cm long pieces and stuck to slides with double-sided tape.
14. To prepare the 'holder' take a small (about 1 cm) glass tube (Hilgenberg catalogue #1413017). Centre it in one end of a microscope slide (parallel to the long side of the slide) in a small droplet (a few μL) of the Araldite/xylene solution and polymerize at 65°C overnight. For the examination, insert the embryo-carrying capillary. Cover the capillary locally with immersion oil and analyse by Nomarski optics. Be careful when you use this device, because the larger capillary in the holder can produce scratches in the microscope objective!!!

Examples of stained embryos embedded according to this protocol are shown in Plate 1C and E.

24.3.6 Staining of imaginal discs in third instar larvae

(a) Dissection

Transfer several third instar larvae (the ones crawling on the wall of the food vial) to an empty Petri dish and wash them with water. Transfer one larva into a siliconized staining dish containing a drop of PBX2. Grasp the larva in the anterior third with two pairs of forceps (Dupont#5) and pull them apart so that the larva is ripped into two pieces. While holding the anterior portion with forceps, take the other forceps to push in the head towards the inside, so that it turns the larva inside out (like a glove finger). Remove gut, salivary glands and fat bodies. Transfer the larval hypodermis, with the imaginal discs sticking to it, to a microfuge tube containing 1 mL PBX2. Keep on ice as long as 30 min while dissecting more larvae following the same protocol.

(b) Fixation

1. After dissection, fix the imaginal discs once by exchanging the PBX2 solution with a premixed solution of 280 μL PBX2 and 120 μL 37% formaldehyde. Add 500 μL heptane. Shake vigorously by hand for 45 s.
2. Fix a second time by exchanging the first fixative with a second premixed solution of 520 μL PBX2, 240 μL 37% formaldehyde and 40 μL dimethyl sulfoxide. Shake gently for 20 min using a rocking device.
3. Wash 2× for 5 min each with 1 mL methanol while shaking the microfuge tube gently.
4. Inactivate the endogenous peroxidases by washing 30 min in 980 μL methanol and 20 μL 30% H_2O_2. After this step, the imaginal discs sticking to the hypodermis can be stored in methanol at –20°C.

(c) Staining

Staining of the imaginal discs is performed as described for the embryos (Section 24.3.3–24.3.5).

(d) Embedding

1. Shake gently for 10 min in 70% ethanol (rocking device).
2. Remove as much 70% ethanol as possible and rinse with 1 mL 100% ethanol.
3. Wash 2× for 10 min in 1 mL 100% ethanol.
4. Wash for 10 min in 1 mL of a mixture of 1:1 100% ethanol/xylene (premix before adding to embryos).
5. Incubate discs for 10 min in 1 mL xylene while rocking the microfuge tube.
6. Put a small drop of DePeX on a slide and transfer one hypodermis preparation with forceps into it. Remove the imaginal discs with forceps and a dissection needle. Discard the remaining tissue. Sink the imaginal disc to the bottom of the drop until it contacts the slide. Put the coverslip on top. Provide slight pressure from the top until the coverslip touches the imaginal disc. Carefully flatten the imaginal disc without disrupting it. Alternatively, if fluorochrome-conjugated antibodies were used for the staining, steps 5 and 6 are modified as follows:

5. Wash the hypodermis preparation 4× in excess PBT.
6. Transfer the hypodermis preparation in a 1:1 mixture of Mowiol/PBS; incubate for 10 min. Dissect out the imaginal discs, discarding the remaining tissue. Transfer the imaginal discs to a drop (10–20 mL) of pure Mowiol and add a coverslip (18 × 18 mm). Wait until Mowiol has hardened.

NOTES

1. Prior to microscopical inspection, distinct portions or tissues of the embryo can be removed with tungsten needles, and thus they can be examined out of the context of the embryo with better optic results. This is the case for 'flat preparations' of the epidermis, with the ventral cord of the nervous system and the muscles attached to it, where the internal organs (fat bodies, gut, etc.) are discarded (Truman and Bate, 1988). Examples of the result of this embedding procedure are shown in Figure 24.1 B, D.
2. The whole-mount preparations are also most suitable for the sectioning of embryos after staining. In *Drosophila*, this technique has been used to visualize proteins required for setting up the dorsal–ventral axis of the embryo and to study the development of the mesoderm (Leptin and Grunewald, 1990).
3. Other methods for localization of proteins in whole mounted tissues of *Drosophila* can be found in Ashburner (1989), McDonald (1994) and Patel (1994).

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CHAPTER TWENTY FIVE

DNA sequencing and primer extension mapping

Hans-Michael Müller

25.1 INTRODUCTION

The two DNA sequencing techniques currently in use are the chemical degradation method developed by Maxam and Gilbert (1979) and the enzymatic method developed by Sanger *et al.* (1977). Due to the less laborious protocol and the easy availability of oligonucleotides, the 'Sanger' method is now the most widely used one, being applicable not only for the sequencing of DNA fragments cloned in plasmid or M13 phage vectors but also, in combination with PCR, for the direct sequencing of minute amounts of template (Ellingboe and Gyllensten, 1992). This chapter describes a standard procedure for the sequencing of single- or double-stranded plasmid DNA, based on the 'Sanger' or 'dideoxy' method.

The principle of dideoxy sequencing is the base-specific termination of DNA polymerase-catalysed primer extension reactions. Four separate DNA synthesis reactions are carried out, each including, in addition to the primer and the four deoxynucleotides, a different chain elongation inhibitor, namely the 2', 3'-dideoxy-derivatives of ATP, GTP, CTP, or TTP. During extension of the primer, these nucleotide analogues are normally incorporated by the DNA polymerase at random positions in the growing DNA strand but the missing 3'-hydroxyl group on the deoxyribose moiety prevents the further elongation of the chain. Each reaction generates a nested set of DNA oligonucleotides with identical 5'-ends and variable 3'-ends, all ending, for example, at each position where an adenosine occurs, when ddATP was used as chain terminator. The elongation products are labelled radioactively by using a ^{32}P end-labelled primer or by including α-^{32}P- or α-^{35}S-deoxynucleotides as internal label. The products of the four reactions, A, G, C, T, are then electrophoresed side-by-side on high-resolution denaturing polyacrylamide gels and visualized by autoradiography. When reading upwards from the bottom of the gel, the sequence of the synthesized strand can be deduced from the sequence in which successively larger fragments occur in the four lanes.

Since the original procedure was published, several changes were introduced to simplify and to improve this method. It was adapted for sequencing of supercoiled plasmid DNA (Chen and Seeburg, 1985), the phosphate label is widely replaced by the less hazardous sulfur label and the highly processive T7 DNA polymerase is now commonly used instead of the *E.coli* Klenow fragment, enabling the determination of longer sequences from a single sequencing reaction.

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DNA sequencing serves not only for sequence determination. The results of the reactions can also be used in marker lanes, e.g. for the determination of the start point of transcription by 'primer extension mapping', the second method described in this chapter. During the primer extension reaction, an oligonucleotide, whose sequence is complementary to the coding strand of the gene and situated approximately 100–150 bases downstream of the suspected start point of transcription, is hybridized to the mRNA and extended until the 5'-end of the mRNA using reverse transcriptase. The same primer is used to perform a sequencing reaction on a corresponding genomic clone, which extends upstream of the presumed start of transcription, at least to the putative TATA-box. Separating the primer extension product and the sequencing reaction side-by-side on a high resolution polyacrylamide gel allows the precise determination of the start point of transcription.

25.2 MATERIALS

25.2.1 DNA sequencing

1. DNA template (Double-stranded DNA: 1.5–2 μg in 32 μL distilled water or TE buffer; single-stranded DNA: 1.5–2 μg in 10 μL distilled water).
2. Sequencing primer (1 pmol/μL).
3. 2 M NaOH.
4. 3 M Sodium acetate, pH 4.8.
5. Ethanol, analytical grade.
6. Ethanol, 80%.
7. α-^{35}S-dATP (10 mCi/mL, >1000 Ci/mMol).
8. T7 Sequencing kit (Pharmacia).

25.2.2 Primer extension mapping

(a) Primer end-labelling

1. Primer (1 pmol/μL).
2. γ-^{32}P-ATP (10 mCi/mL, >5000 Ci/mmol, 2 pmol/μL).
3. Phosphorylation buffer (Boehringer).
4. Polynucleotide kinase, 10 U/μL (Boehringer).

(b) Primer extension

1. 5× hybridization buffer: (1 M KCl, 50 mM Tris/HCl, pH 8.3).
2. 5× Reverse transcriptase buffer (50 mM $MgCl_2$, 100 mM Tris-HCl, pH 8.3).
3. 200 mM DTT.
4. 10 mM dNTP solution.
5. RNase inhibitor, from human placenta, 40 U/μL (Boehringer).
6. AMV-reverse transcriptase, 25 U/μL (Boehringer).
7. 500 mM EDTA pH 8.0.
8. RNaseA (Boehringer): dissolve in distilled water (10 mg/mL), boil for 10 min, store at –20°C.
9. TE buffer: 1 mM EDTA, 10 mM Tris-HCl, pH 8.0.

10. 5 M NaCl.
11. Phenol.
12. Chloroform/isoamyl alcohol (24:1).
13. Ethanol, analytical grade.
14. 80% Ethanol.
15. Stop-buffer (from T7 Sequencing Kit).

25.2.3 Gel electrophoresis

1. Glass plates, 4–6 mm × 20 cm × 40 cm.
2. Spacers and combs (0.4 mm).
3. Dimethyl-dichloro-silane (Sigma): prepare 4% stock in chloroform, store at RT.
4. γ-Methacryl-oxypropyl-trimethoxy-silane (Sigma), add 30 μL to 300 μL 10% acetic acid in 10 mL ethanol, prepare fresh.
5. Ethanol, technical grade.
6. 10× Tris-borate buffer (TBE): 900 mM Tris base, 900 mM boric acid, 30 mM EDTA, pH 8.3.
7. 10% acrylamide/N,N'-methylenebisacrylamide (20:1), 8 M urea, in TBE. Filter and store at 4°C, protected from light.
8. 8 M urea in TBE, filter and store at 4°C.
9. Ammonium persulfate (APS), Sigma.
10. N,N,N',N',-Tetramethyl-ethylendiamine (TEMED), Merck.
11. Electrophoresis buffer: 1× TBE.
12. Fixing solution (10% acetic acid/10% methanol in distilled water).
13. X-ray films (Kodak, X-OMAT AR).

Where not indicated, analytical grade reagents are used without special preference for supplier. All reagents are stored as recommended by the supplier.

25.3 METHODS

25.3.1 DNA sequencing

The following protocol is designed for sequencing of DNA fragments cloned in plasmid or M13-derived cloning vectors. The high copy number phagemid pBluescript is widely used for this purpose, as it allows the isolation of plasmid DNA in double-stranded supercoiled conformation but also as a single-stranded product upon infection with bacteriophage f1 (Sambrook *et al.*, 1989). 'Universal primers' flanking the cloning sites in the polylinker allow the sequence determination of any cloned DNA fragment using the dideoxy method. Inserts longer than 1 kb may be subcloned as smaller fragments, or internal primers may be designed to complete the sequence determination. For template preparation, many sophisticated commercial kits are now available, but simple DNA extraction protocols like the alkaline lysis method (Birnboim and Doly, 1979) also lead to good results (Figure 25.1). In the protocol given below, sequencing reactions are performed using the T7 Sequencing Kit of Pharmacia. As T7 DNA polymerase uses dideoxynucleotides very readily, these are excluded during the first stage of the sequencing reaction, the labelling reaction, and

added to the second stage, the termination reaction, in order to permit synthesis of long chain-terminated fragments. For routine sequencing, the determination of sequences up to 400 bases (double-stranded template) or up to 600 bases (single-stranded template), respectively, the 'short' sequencing mixes (provided in the kit) are used. All solutions necessary are provided in this kit with the exception of the radioactive label and the solutions used for denaturation of double-stranded template. As radioactive label we recommend α-^{35}S-dATP or α-^{35}S-dCTP since this isotope is less hazardous then ^{32}P. *However, use all standard protection during handling of the radioactive samples and ensure the safe disposal of the radioactive waste according to the local regulations.*

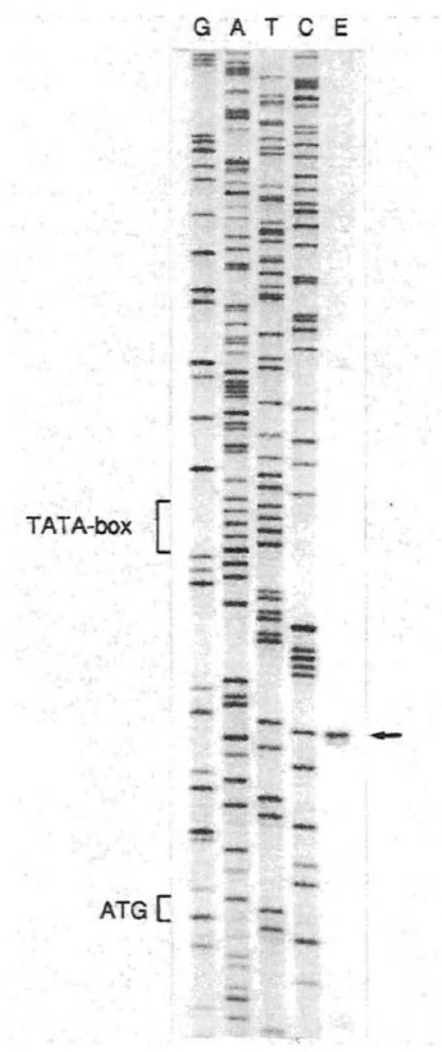


Figure 25.1 Identification of the transcription start point of the *An. gambiae* trypsin gene *Antryp4*. Primer extension was done using total RNA extracted from female mosquitoes using a ^{32}P-end-labelled 24mer-oligonucleotide. A genomic subclone containing the upstream region and the proximal part of the coding region of *Antryp4* was sequenced with the same primer, using supercoiled DNA template and internal ^{35}S-labelling. Sequencing reactions (GATC) and primer extension product (E) were separated on a 6% denaturing polyacrylamide gel. The TATA-box and the start-codon are indicated. The position of the cap site is shown by an arrow. GATC indicate the coding strand.

(a) Denaturation of double-stranded template

1. Adjust template concentration to 1.5–2 μg/32 μL with distilled water.
2. Add 8 μL 2 M NaOH and mix.
3. Incubate at RT for 10 min.
4. Put on ice and add 4 μL of distilled water and 7 μL 3 M sodium acetate, pH 4.8.
5. Add 120 μL of ethanol, mix by vortexing, and leave for 15 min at –20°C; centrifuge for 20 min.
6. Remove supernatant and carefully add 200 μL 80% ethanol. Do not vortex. Centrifuge for 10 min, remove supernatant and dry the pellet briefly under vacuum.

(b) Primer annealing

1. Resuspend the DNA pellet prepared according to 3.1 in 10 μL sterile water.
2. Add to the denatured template, or to 10 μL single-stranded template (1.5–2 μg), 2 μL primer (1 pmol/μL) and 2 μL of annealing buffer, mix by vortexing and spin briefly.
3. Incubate at 37°C for 20 min (in the case of double-stranded DNA) or at 60°C for 10 min (for single-stranded DNA templates). From this point on, both types of template are treated equally.
4. Cool the annealing reaction for 10 min at RT and spin briefly.

(c) Labelling reaction

1. While the annealing reaction proceeds, label four microfuge tubes or microtitre plate wells with A,G,C,T and add 2.5 μL of the A,G,C,T *'short'-sequencing mixes* into the corresponding tube or well.
2. Prepare the labelling enzyme premix by adding in a microfuge tube on ice: 1 μL of sterile water, 3 μL of the A-labelling mix (dGTP, dCTP, dTTP), 2 μL of T7 DNA polymerase (diluted 1:5 in enzyme dilution buffer) and 1 μL of α-^{35}S-dATP. Mix gently and keep on ice. Scale up this mix according to the number of reactions.
3. Add 6 μL of the labelling enzyme premix to the annealing reaction (Section 25.3.1 b, step 4) and incubate at RT for 5 min.

(d) Termination reaction

1. During the labelling reaction, prewarm the sequencing mixes (3.1.3 step 1) at 37°C for at least 1 min.
2. When the labelling reaction (Section 25.3.1 c, step 3) has been incubated for 5 min, transfer aliquots of 3.5 μL into each of the prewarmed A,G,C,T sequencing mixes, using a new pipette tip for each reaction. Mix gently and incubate for further 5 min at 37°C.
3. Add 5 μL of stop solution to each tube and store on ice. If not used the same day, store the samples at –20°C.

25.3.2 Primer extension

Although the use of purified mRNA is preferable, the method given below also works well with total RNA preparations. The RNA used for the experiment shown in Figure

25.1 was extracted from *Anopheles gambiae* mosquitoes using the proteinase K-method (Sambrook *et al.*, 1989), modified as described (Müller *et al.*, 1993). Contaminating chromosomal DNA was separated by precipitating the RNA three times in 4 M LiCl. The extension product is analysed by running it side-by-side with a sequencing reaction performed with the same primer and a genomic clone which should extend at least to the putative TATA-box. The primer used for the extension reaction, but not necessarily for the sequencing reaction, is end-labelled with ^{32}P, *therefore ensure appropriate safety measures.*

(a) Primer end-labelling

1. In a microfuge tube, add 2 μL sterile water, 0.5 μL PNK buffer (provided with the enzyme by the manufacturer), 1 μL of primer (1 pmol), 1 μL of γ-^{32}P-ATP (2 pmol), and 0.5 μL polynucleotide kinase.
2. Incubate at 37°C for 30 min.
3. Stop the reaction by heating to 65°C for 5 min.
4. Spin down the samples and store at –20°C. Removal of unincorporated γ-^{32}P-ATP is not necessary.

(b) Primer annealing and extension

1. 6–7 μg of total RNA (this corresponds to one blood-fed or three nonfed female *An. gambiae* mosquitoes) are ethanol precipitated, the pellet washed with 80% ethanol and air-dried.
2. The RNA is dissolved in 6 μL sterile water, and 2 μL primer (0.4 pmol) and 2 μL 5× hybridization buffer are added. After denaturation for 3 min at 85°C, samples are annealed for 1 h at 55°C.
3. A few minutes before the annealing step is finished, the reverse transcription mix is prepared on ice: 14 μL sterile water, 8 μL 5× reverse transcription buffer, 2 μL 200 mM DTT, 4 μL 10 mM dNTP, 1 μL RNasin, 1 μL AMV-reverse transcriptase. Scale up according to the number of primer extension reactions.
4. Briefly spin down the annealing reaction, place it at 42°C and add 40 μL of the reverse transcription mix. Incubate for 60 min at 42°C.
5. Briefly spin down and stop the reactions by adding 2 μL 500 mM EDTA.
6. Add 1 μL RNaseA and incubate for 10 min at 37°C.
7. Add 156 μL TE buffer and 4 μL 5 M NaCl.
8. Add 200 μL phenol/chloroform (1:1), mix by vortexing, centrifuge for 10 min, transfer the upper phase to a new microfuge tube.
9. Add 600 μL ethanol (do not add more salt!), mix, place for 30 min at –20°C and centrifuge for 20 min at maximum speed.
10. Carefully remove supernatant and slowly add 200 μL 80% EtOH, without disturbing the pellet (do not vortex!) and centrifuge 10 min at maximal speed. Carefully remove supernatant and air-dry the pellet.
11. Rinse the wall of the microfuge tube with 4 μL of TE buffer, then place the drop of TE on the pellet. Add in the same way 6 μL of stop buffer (available from the sequencing kit). Dissolve the pellet by pipetting up and down; use a Geiger monitor to ensure that only a minor part of the radioactivity remains in the tip.
12. If not used the same day, store the samples at –20°C, otherwise put them on ice.

25.3.3 Gel electrophoresis

The sequencing reactions and the primer extension reactions are analysed on denaturing polyacrylamide gels. The time during polymerization and prerun of the gel can be used to perform the sequencing reactions.

(a) Plate treatment

Before assembling, pouring and polymerization of the acrylamide, the notched-plate and the front-plate are treated with two different silane-derivatives, dimethyl-dichloro-silane and γ-methacryl-oxypropyl-trimethoxy-silane, respectively. The first one, the 'repel-silane', prevents the strong attachment of the acrylamide gel to the notched-plate, and therefore facilitates the separation of this plate from the gel after electrophoresis. The second mediates the attachment of the gel to the front-plate, via covalent bonds which form during polymerization of the acrylamide. This treatment allows the gel to be fixed and dried directly on the glass plate, to which the X-ray film is then exposed. *Work under the hood and wear gloves, as silane is toxic.*

1. Clean both plates with soap and rinse them well, first with tap water then with distilled water. Dry them with a paper towel, rinse them with ethanol and dry them again with a paper towel. Both plates are then silanized under a chemical hood, using gloves. It is important to treat one plate after the other, never placing both plates together under the hood, as in this case the very volatile silanes would cross contaminate the plates. Leave all paper towels used for silanization in the hood until they are dry before disposing of them.
2. Place the notched-plate under the hood and pour 10 mL of repel-silane (4% in chloroform) onto it. Using a paper towel, distribute it until most of the solution has evaporated. This will take a few minutes. Leave the treated plate for a further 5 min under the hood, then remove excess repel-silane with a wet paper towel, rinse the plate with distilled water and dry.
3. Place the front-plate under the hood and pour 10 mL of freshly prepared γ-methacryl-oxypropyl-trimethoxy-silane solution on it. Distribute the solution equally on the plate, as described for the 'repel'-silane, and leave a further 10 min under the hood. Remove the excess of silane with an ethanol-wetted paper towel. Repeat this step four times, each time using a fresh paper towel, then leave the dry plate for another 10 min under the hood.

(b) Gel preparation

The use of two urea/TBE stock solutions, of which only one contains acrylamide (10%), allows the percentage of the individual gels to be varied, without the need to prepare several acrylamide stock solutions in advance. This also facilitates fine adjustments of the gel percentage, when a new batch of acrylamide is used, especially when the supplier of the acrylamide has been changed. For routine sequencing, a 5% gel is most appropriate. If an optimal resolution around the 100 bp area or below is desired, for example when running a primer extension sample in parallel, a 6% gel is recommended. *Wear gloves and avoid mouth-pipetting, as acrylamide is very toxic.*

1. Assemble the glass plates with the silane-treated surface facing inward, placing the spacers between the long and the bottom edges and fix the plate-assembly with clamps.

2. For a 5% gel, mix 20 mL of the urea/TBE and 20 mL of the 10% acrylamide/urea/TBE stock solution in a beaker containing a magnetic bar. During mixing on a magnetic stirrer, add 80 μL of TEMED and 160 μl of 10% APS. Continue stirring for a few seconds, then immediately begin pouring the gel.
3. Hold the assembled plates at an angle of 45°, one corner of the bottom resting on the bench. Fill a 30 mL glass pipette with the acrylamide solution and pour it between the plates by injecting it continuously at one corner of the setup. Take care not to interrupt the flow of the acrylamide solution, in order to avoid the formation of air bubbles. After pouring the gel, lay the plates in a horizontal position, and insert the comb. When using a shark-tooth comb, insert it upside down, a few millimetres less deep than the length of the teeth. Let the gel polymerize for at least 30 min. It is often convenient to prepare the gel the day before it is used. In this case, wet the area around the comb with some drops of distilled water and cover the top of the gel with plastic film.

(c) Sample loading and electrophoresis

1. Gently bend out the bottom spacer, then remove it.
2. Wet the comb area with distilled water, and slowly remove the comb. Immediately rinse the sample wells with distilled water to remove unpolymerized acrylamide.
3. Place the gel in the electrophoresis apparatus and add running buffer (1× TBE), when using a shark-tooth comb, insert it so that the points just touch the gel surface.
4. Clamp an aluminium plate on the front-plate, taking care that it does not extend into the buffer of the lower reservoir. The aluminium plate ensures an equal heat distribution, thus minimizing the 'smiling' effect.
5. Mark on the plate, using a water-proof pen, groups of four wells for each set of sequencing reactions. Allocate further adjacent groups of four wells when multiple runs of the same sequencing reaction are planned. Prerun the gel at 40 mA for 1 h. During the prerun, the gel should have reached a temperature of ~50–55°C.
6. Prior to loading, denature the samples of the sequencing or primer extension reactions by placing the tubes at 90°C for 3 min, then put them on ice in the order in which the samples will be loaded.
7. Switch off the power supply and wash out the wells using a micropipette. This washing step is important, as the urea having diffused from the gel into the sample wells will prevent a proper loading and an optimal focusing of the bands. When using a conventional comb, wash and load only a few wells at a time, as the urea rapidly diffuses back into the wells. Load 2 μL of each sequencing reaction in adjacent wells, always using a fixed order, for example A,G,C,T. Switch on the power supply and continue electrophoresis at 40 mA. Stop electrophoresis when the bromophenol blue reaches the bottom of the gel.
8. When performing three overlapping runs with the same sequencing reaction, it is possible to read 400–500 bases of sequence. For overlapping runs, continue electrophoresis until the xylene cyanol is 10 cm from the bottom of the gel. Then switch off the power supply, wash out the wells and load an aliquot of the same set of reactions in four adjacent wells. After the last loading, continue elec-

trophoresis until the bromphenol blue has reached the bottom of the gel. For the triple, double and single run load 4 μL, 3 μL and 2 μL, respectively; this will increase the signal strength of the otherwise relatively faint high molecular weight bands.

(d) Autoradiography

1. Switch off the power supply and remove the gel-plate assembly from the electrophoresis apparatus, then place it in the sink and rinse the notched-plate with cold water. Due to the temperature difference, the notched-plate will soon detach from the gel.
2. Put the front plate with the attached gel in a tray containing 2 L of fixing solution. Leave the gel submerged for about 15 min, agitating a few times during the period. When the colour of the bromophenol blue changes to yellow, the fixation is finished. The fixing solution can be reused up to 20 times.
3. Put the plate in the sink and wash the gel with tap water for 10 min. As the acrylamide is covalently bound to the glass plate, substantial water pressure can be applied without damaging the gel.
4. Dry the back and the edges of the plate and dry the gel in a microwave oven; set on half power, this will take 10–15 min. Drying in the microwave oven is recommended only when glass plates of 6 mm thickness are used. Alternatively, dry the gel in an oven at 80°C.
5. When the plate with the dried gel has cooled down, put it in a film cassette, then put an X-ray film on the gel and expose overnight at RT.
6. Develop the X-ray film according to the manufacturer's instructions.
7. After film exposure, detach the gel from the glass plate by submerging it overnight in a tray containing 2 L of 2% KOH in distilled water. The KOH solution may be reused until it is exhausted.

NOTES

Sequencing – some problems which may occur

1. The bands are faint. The reason for this may be that the label used was too old, or the template or primer concentration was too low. Weak sequences occur often when an insert-specific primer was synthesized. When designing such primers, check that they do not contain palindromic sequences which reduce the rate of hybridization to the template. You may further optimize the signal strength by using radioactive dATP or dGTP as internal label, if the DNA insert to be sequenced is A/T- or G/C-rich, respectively.
2. The bands adjacent to the primer are faint or absent. This problem often occurs with self-designed primers. Ensure that the complementary sequence near the 3′-end of the sequencing primer contains some T residues when using radioactive dATP as internal label. In this case, the sequence may be readable as little as 10 bases upstream to the primer.
3. The bands are diffuse or smeary. This might be due to sample overloading, overheating of the gel during electrophoresis, or dirty combs and glass plates. A further reason could be that unpolymerized acrylamide was not washed out

immediately after removing the comb, or that the urea was not washed out from the wells before sample loading. The problem could also be due to the presence of impurities in the TBE running buffer or the acrylamide solution. Filter the solutions and try again. If this does not lead to an improvement, prepare fresh solutions.

4. You observe artefactual bands in all four lanes throughout the whole sequence. The most common reason for this is poor quality of the template. Trying to improve the template quality by additional phenol extractions and ethanol precipitations usually does not solve the problem, it is best to prepare the template again. Another reason might be the use of too little DNA polymerase, or degradation of the DNA polymerase.
5. There are artefactual bands that can only be due to the contamination of the template with a 'background' clone lacking an insert. Pick only well separated colonies for the inoculation of overnight cultures. Even slight contamination with a 'background' colony may have a detrimental effect, as the cells harbouring a plasmid without insert often have a growth advantage.
6. You observe strong stops at all four lanes at a defined position. The template may form strong secondary structures that cause the DNA polymerase to pause. Carrying out the reactions with *Taq* DNA polymerase at a higher temperature may help (Ellingboe and Gyllensten, 1992). Also sequencing the complementary strand often solves this problem.
7. Band compressions are generated when fragments differing by one or a few nucleotides migrate with similar mobilities, usually because G residues have formed secondary structures which were not denatured during electrophoresis. Increasing the gel temperature during electrophoresis to 60°C helps in many cases; you may control the gel temperature using thermo-regulated glass plates. Alternatively, use sequencing mixes containing the nucleotide analogue 7-deaza-dGTP, which prevents G–G base aggregation (Deaza Sequencing Mixes, Pharmacia).

Primer extension

1. Usually, not all of the sample of a primer extension reaction is used for the first electrophoresis. Use the information provided by the autoradiograph of the first gel to adjust the time of the second electrophoresis in order to achieve an optimal resolution of the sequence ladder near the extension product, by using the relative positions of the extension product and the xylene cyanol as marker. In Figure 25.1, the electrophoresis was stopped when the distance between xylene cyanol and the bottom of the gel was 7 cm. The extension product had a size of 124 bases, and migrated 1 cm above the xylene cyanol. In order to make the result as convincing as possible, also load in this second run the sequencing reactions in such a way that the sequencing and the reverse transcribed extension products of identical size are running side-by-side (Figure 25.1).
2. The extension product does not co-migrate with any band of the sequence ladder. If internally-labelled sequencing reactions are used as marker lanes for primer extensions which yield extension products <90 bp, the primer should be end-labelled with 'cold' ATP in order to avoid misinterpretations. When sequence reactions are performed with unphosphorylated primers, the bands in

the 70 base area will migrate up to one base slower with respect to the end-labelled primer extension product. For end-labelling of the sequencing primer, use the same method as described in Section 25.3.2a using instead 10 pmol of primer and 1 μL ATP (10 μM) in a final volume of 10 μL. The PNK-buffer does not disturb the sequencing reaction.

3. There is an additional band one base below the extension product (Figure 25.1). This may be due to a multiple start site of transcription, but commonly this is due to the 'Cap effect': A full-length reverse transcript of a eukaryotic mRNA is often accompanied by a minor reverse transcript, one nucleotide shorter than the full-length transcript. This is thought to be due to premature termination of reverse transcription at the methylated residue situated next to the cap site. We observed that the intensity of this minor band may vary when using AMV reverse transcriptase from different suppliers. Often this problem can be resolved, at least partially, by increasing the amount of the reverse transcriptase used in the reaction.
4. You observe several bands at various positions below the full-length extension product. These may have been generated by multiple transcriptional starts, mRNA secondary structures which provoke pausing of the reverse transcriptase, the primer being too far from the 5'-end of the mRNA, or the primer cross-hybridizing to related (but different) mRNAs. Analyse the proximal part of the coding region for secondary structures and design a primer hybridizing upstream of these structures; the optimal distance between putative transcription start site and primer being 70–150 bases. If you still observe the same banding pattern with the second primer, this indicates multiple start sites, as cross-hybridization is usually improbable. Elevating the hybridization temperature or sequencing directly the extension product, by including dideoxy nucleotides in the reaction, are further ways to exclude cross-hybridization. If the gene of interest is a member of a multi-gene family of which the gene sequences are known, design the primers in a way to minimize cross-hybridization. Figure 25.1 shows the determination of the transcription start point of *Antryp4*, a member of seven trypsin genes in *An. gambiae* (Müller *et al.*, 1993). The primers were designed according to a short polymorphic region. The corresponding gene cluster is sequenced and we observed 30 bases downstream of the centre of the conserved TATA-boxes a conserved sequence block, containing the putative start site. We designed the primers for all seven genes in such a way, that the expected extension products, 110–124 bases in length, differed by two bases each, thus providing an additional specificity control.

Gel electrophoresis and audioradiography

1. The glass plates should have a thickness of at least 4 mm; thicker plates (6mm) are preferable as they keep retain the temperature of the prerun gel better during sample loading and lead to a better heat distribution during electrophoresis, especially in combination with an aluminium plate, thus minimizing the 'smiling' effect.
2. An alternative method to the fixation of the gel on the front-plate is the transfer of the fixed gel onto filter paper. In this case, only the notched-plate is treated with repel-silane and the front-plate is not treated. After fixation, the gel is trans-

ferred onto filter paper, then covered with plastic wrap, and dried in a vacuum gel dryer. Prior to film exposure the plastic wrap is removed. However, using this method the gel has to be handled very carefully during fixation, as it easily detaches from the glass plate.

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Part Four
Genome Mapping Techniques

CHAPTER TWENTY SIX

RFLP analysis of insect genomes

David W. Severson

26.1 INTRODUCTION

Genetic markers are useful for identifying and tracking genes in segregating populations and for comparing genetic diversity among populations. Traditionally, genetic linkage maps were generated by combining data from many populations, because only a limited number of markers segregated in any one population. Restriction fragment length polymorphism (RFLP) analysis employs cloned DNA sequences that hybridize to specific regions of the genome, with genetic polymorphisms defined as differences in the length of DNA fragments observed following digestion with restriction endonucleases and Southern blotting procedures. Since the demonstration of their utility for genetic mapping (Botstein *et al.*, 1980), RFLP markers have been widely used for constructing comprehensive linkage maps for many plant and animal species.

RFLP analyses of insects can be difficult because of their small size and their correspondingly small amount of total genomic DNA. Still, useful Southern blots can be prepared from insects with as little as 0.5 μg of extractable DNA per individual. With these smaller insects, RFLP analysis is generally limited to a single restriction enzyme digest and a single Southern blotting effort for each individual. The individual Southern blots can, however, routinely be regenerated for 15–25 rounds of hybridization. A detailed RFLP linkage map exists for the yellow fever mosquito, *Aedes aegypti* (Severson *et al.*, 1993), and these markers have been used to identify genome regions carrying genes determining mosquito competence to transmit some malarial (Severson *et al.*, 1995a) and filarial worm (Severson *et al.*, 1994; Beerntsen *et al.*, 1995) parasites to their vertebrate hosts. With *Anopheles gambiae* mosquitoes, Romans *et al.* (1991) used RFLP markers to investigate the genetic relationship between a putative diphenol oxidase gene and an esterase locus previously associated with resistance to some malarial parasites. RFLP markers have also been linked with insecticide resistance in the German cockroach, *Blattella germanica* (Dong and Scott, 1994), and with genes for diapause hormone and a neuropeptide in the silkmoth, *Bombyx mori* (Pinyarat *et al.*, 1995).

RFLP markers do offer several advantages for genome analysis in insects. We prefer to develop cDNA clones as RFLP markers because they typically represent single or low copy number sequences for which putative gene functions can frequently be determined with partial sequence information (Severson and Zhang, 1996).

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Transcribed sequences also reflect sufficient evolutionary conservation, even among distantly related species, for developing comparative linkage maps. Comparative maps, based on common RFLP markers, have been developed for maize with rice (Ahn and Tanksley, 1993), sugarcane (D'Hont *et al.*, 1994, and sorghum (Pereira *et al.*, 1994), for tomato with potato (Bonierbale *et al.*, 1988) and pepper (Tanksley *et al.*, 1988), for garden pea with lentil (Weeden *et al.*, 1992), for human with mouse (Nadeau *et al.*, 1992) and for the mosquito, *Aedes albopictus* with *Ae. aegypti* (Severson *et al.*, 1995b). The methods described here were developed for use with *Ae. aegypti* and should, therefore, be applicable to most insect species of this general size or greater.

26.2 MATERIALS

26.2.1 DNA isolation and purification

1. DEB lysis buffer: 1% SDS, 0.2 M NaCl, 25 mM EDTA, 10 mM Tris, pH 8.0 (prepare fresh).
2. Proteinase K: 20 mg/mL (store at 4°C).
3. RNase A: 10 mg/mL (store at –20°C; Sigma R-5125).
4. 1 M Tris, pH 9.5.
5. Phenol: Equilibrated in 1 M Tris, pH 9.5 containing 0.1% 8-hydroxyquinoline (store in dark at –20°C; GibcoBRL #15509-011) (*avoid contact with skin and wear safety glasses*).
6. 8-Hydroxyquinoline: (Sigma H-6878).
7. Chloroform.
8. 95% Ethanol.
9. Restriction enzyme and appropriate buffer.
10. BSA: 1 mg/mL (GibcoBRL 11018-025).
11. Spermidine: 100 mM (Sigma S-2501).
12. Plastic pellet pestles: (Kontes #749520-0000).
13. Microcentrifuge tubes: 0.5 and 1.5 mL.

26.2.2 DNA separation

1. Horizontal slab gel apparatus and power supply.
2. Agarose: (TreviGel™ 5000; Trevigen #9806-50-P).
3. 50× TAE buffer: 242 g Tris, 57.1 mL glacial acetic acid, 100 mL 0.5 M EDTA, pH 8.0). Adjust volume to 1 L with ddH_2O.
4. Ethidium bromide: 10 mg/mL (store in dark at RT). *This is a mutagen, avoid contact with skin.*
5. Agarose dye: 0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol in ddH_2O.
6. UV transilluminator.

26.2.3 Southern blotting with nylon membranes

1. Gel denature solution: 150 mM NaOH, 3 mM EDTA, pH 8.0.
2. Gel neutralize solution: 150 mM sodium phosphate buffer, pH 7.8 (below).

3. Sodium phosphate buffer stock solution: 0.5 M, pH 7.8.
4. Sodium pyrophosphate stock solution: 0.1 M (Sigma P-8010).
5. Whatman chromatography paper #3030917.
6. 254_{nm} germicidal lamp.
7. Nylon membrane, positively charged.

26.2.4 DNA insert preparation

1. *Taq* DNA polymerase with 10× reaction buffer.
2. 25 mM $MgCl_2$.
3. 5 mM dNTP mix.
4. Primers: Specific primers at ~10 ng/μL (1.5 μM).
5. Mineral oil.
6. Chloroform.
7. Thermal cycler.
8. Low melting point agarose (GibcoBRL #15517-014).
9. Phenol.
10. 4 M Ammonium acetate.
11. 95% Ethanol.
12. 70% Ethanol.
13. TE, pH 7.4: 10 mM Tris, pH 7.4, 1 mM EDTA.

26.2.5 Probe radiolabelling

1. *Taq* DNA polymerase with 10× reaction buffer.
2. 25 mM $MgCl_2$.
3. 5 mM dATP, dGTP, dTTP mix.
4. Primers: Specific primers at ~10 ng/μL (1.5 μM); Random nine-mer at 1 mg/mL.
5. Thermal cycler.
6. $\alpha^{32}P$-dCTP: (3000 Ci/mMole, Dupont NEN-013H).
7. Biogel elution buffer: 0.5% SDS, 1 mM EDTA, 20 mM Tris, pH 7.4, 5 μg/mL yeast RNA.
8. Biogel suspension: Slowly add 1 g Biogel P60 (Bio-Rad #150-4160) to 28 mL Biogel elution buffer using a stirplate (this equals about 10 bed volumes of buffer). Allow to equilibrate for several hours (preferably overnight) before use. Store at 4°C.
9. Yeast RNA: 10 mg/mL (Sigma R-6625).
10. Orange G: 0.5% (w/v) Orange G in ddH_2O (Sigma O-7252).

26.2.6 λ *Hin*dIII marker radiolabelling

1. λ phage DNA: (Boehringer Mannheim Biochemicals #745-782).
2. *Hin*dIII restriction enzyme and 10× reaction buffer.
3. Phenol.
4. Chloroform.
5. 95% Ethanol.
6. 70% Ethanol.

7. TE pH 8.0: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA.
8. 10× Nick translation buffer: 0.5 M Tris, pH 7.4, 0.1 M $MgSO_4$, 1 mM DTT, 0.5 mg/mL BSA, 0.2 mM each dATP, dGTP, dTTP (store at –20°C).
9. α^{32}P-dCTP: (3000 Ci/mMole, Dupont NEN-013H).
10. Klenow (DNA polymerase I large fragment).

26.2.7 Blot hybridization and regeneration

1. Blot preblock solution: 2% SDS, 0.5% BSA, 1 mM EDTA, 1 mM *o*-phenanthroline.
2. Hybridization solution: 0.1 M $NaPO_4$, pH 7.8, 20 mM sodium pyrophosphate, 5 mM EDTA, 0.1% SDS, 10% (w/v) sodium dextran sulfate, 1 mM *o*-phenanthroline, 0.5 mg/mL heparin sulfate, 50 µg/mL sheared salmon sperm DNA, 50 µg/mL yeast RNA. To prepare 100 mL: Warm about 30 mL ddH_2O, the sodium phosphate buffer and the sodium pyrophosphate in a beaker on a hot plate at a medium setting with stirring. Gradually add the sodium dextran sulfate until completely in solution. Turn off heat and add remaining ingredients. Bring to a final volume of 100 mL with ddH_2O. Store at 4°C.
3. 1 M *o*-Phenanthroline: prepare in 95% ethanol; Sigma P-9375.
4. Sodium dextran sulfate: (Dextran Products Ltd, Scarborough, Ontario, Canada.).
5. Heparin sulfate: (Sigma H-7005).
6. Sodium phosphate buffer stock solution: 0.5 M, pH 7.8.
7. Sodium pyrophosphate stock solution: 0.1 M (Sigma P-8010).
8. Salmon sperm DNA: (Sigma D-1626). To prepare: Dissolve 1 g of DNA in 100 mL 0.1 M NaCl. Extract with phenol/chloroform. Shear DNA by drawing supernatant through an 18½ G needle about 12 times. Precipitate with ethanol and resuspend in ddH_2O. Check concentration by spectrophotometer A_{260} readings and adjust to final concentration of about 10 mg/mL. Store in aliquots at –20°C.
9. Yeast RNA: 10 mg/mL (Sigma R-6625).
10. 20×SSC: (1×SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7.0).
11. 10% SDS.
12. Wash solution I: 2×SSC, 0.1% SDS.
13. Wash solution II: 0.2×SSC, 0.1% SDS.
14. Hybridization oven.
15. Plastic sheet savers: (20th Century Plastics #NRPB30-00).
16. X-ray film: (Kodak XAR-5).
17. Staticide: (ACI Inc. #2005).
18. 0.4 N NaOH.
19. Blot neutralizer solution: 0.1×SSC, 0.1% SDS, 0.2 M Tris, pH, 7.4.

26.3 METHODS

26.3.1 DNA isolation and purification

This method was developed to produce DNA suitable for restriction enzyme digestion with a minimum of handling and is amenable to rapidly processing large numbers of individual mosquitoes (Severson and Kassner, 1995). It can be used to isolate genomic DNA from fresh, frozen or ethanol-preserved insects. With ethanol-pre-

served tissue, blot the individuals on paper towelling to remove excess ethanol, then allow them to air-dry overnight at RT before proceeding (D. Zaitlin, unpublished).

1. In a 1.5 mL microcentrifuge tube and using a plastic pellet pestle, homogenize a single mosquito in 120 μL of freshly prepared DEB and 1.5 μL of RNase A. With ethanol preserved individuals, grind dry to a powder, add the DEB and RNase A, then rehomogenize. Incubate at 37°C for 60 min.
2. Add 3 μL of proteinase K and incubate at 50°C for 60 min.
3. Add 60 μL each of phenol and chloroform. Vortex and centrifuge at full speed in a microfuge for 10 min.
4. Transfer the supernatant to a 0.5 mL microcentrifuge tube and add 300 μL of 95% ethanol. Use care to avoid transferring any of the organic phase as restriction enzymes are extremely sensitive to inactivation by phenol. Mix well by laying tubes horizontal on a platform shaker and shaking for 10–15 min. Incubate at –20°C overnight.
5. Centrifuge at full speed in a microfuge for 10 min. Discard supernatant, remove as much ethanol as possible with a 10 μL micropipette. Incubate tubes at 37°C for ~10 min with the cap open to evaporate remaining ethanol.
6. Resuspend DNA pellet in 11.1 μL ddH_2O. Incubation at 65°C will facilitate DNA resuspension. The DNA must be completely resuspended or it will not digest with restriction enzymes.
7. Restriction digests can be set up from a master mix containing for each DNA preparation: 0.2 μL restriction enzyme (1–2 U), 1.5 μL 10× reaction buffer, 0.1 μL RNase A, 1.5 μL BSA, and 0.6 μL spermidine. Add 3.9 μL of the master mix to each tube and incubate at 37°C for 1 h minimum. After incubation, proceed with DNA separation (Section 26.3.2) or store at –20°C.

26.3.2 DNA separation

1. Prepare a 0.9% agarose gel of ~5 mm thickness. We use a fine-toothed comb to provide a well capacity of ~20 μL. This concentrates the DNA in a small area and enhances resolution of restriction fragments, particularly when small quantities of DNA are size-fractionated.
2. Add 1/10 vol agarose dye to each restriction digest. Mix well and centrifuge briefly to pellet sample.
3. Load samples into gel. Load λ *Hin*dIII marker DNA at 15–20 well intervals. Perform electrophoresis in 1× TAE buffer with buffer recirculation at ~1 V/cm until the bromophenol blue dye just runs off the gel.
4. Following size fractionation, stain the gel for ~1 h with 0.5 μg/mL EtBr in 1× TAE buffer, followed by a ~1 h destain in ddH_2O. Visualize the DNA under UV light and photograph the gel for a permanent record.

26.3.3 Southern blotting with nylon membranes

The general method of Southern (1975) remains as the procedure of choice for permanently transferring DNA restriction fragments to a solid matrix (Chapter 20). Various modifications of the procedure are currently in practice. However, for maximizing the amount of information obtainable from individual Southern blots, the use

of positively charged nylon membranes and capillary transfer of the DNA with an alkaline buffer (Reed and Mann, 1985) is preferred. This procedure covalently binds the DNA to the membrane. The method described here is adapted from the literature (Murray *et al.*, 1992; Zaitlin *et al.*, 1993) and routinely provides 15–20 rounds of hybridization on membranes carrying genomic DNA digests from individual mosquitoes. Additionally, 2 or 3 different probes can frequently be hybridized simultaneously to the same blot, to increase the total number of markers evaluated for each individual (Severson *et al.*, 1993).

1. Place the agarose gel carrying the size-fractionated genomic DNA about 6–7 cm from a 254_{nm} germicidal lamp with the high-molecular weight end of the gel centred directly under the lamp. Photo-nick the DNA by exposure to the light for 1 min.
2. Transfer the gel to a clean plastic or glass container with sufficient gel denature solution to cover the gel. Shake gently at RT on a platform shaker for 15 min (minigels) or 25 min (larger gels). Carefully pour off the gel denature solution and rinse the gel 3× with ddH_2O.
3. Add sufficient gel neutralizer solution to cover the gel. Shake gently at RT on a platform shaker for 20 min (minigels) or 30 min (larger gels).
4. Cut a piece of nylon membrane exactly the size of the gel and prewet in 25 mM sodium pyrophosphate for at least 15 min before setting up the transfer. Additionally, cut 6 pieces of Whatman filter paper and a 4–5 inch stack of single-fold paper towels the same size as the gel.
5. Set up transfer of the DNA from the gel to the nylon membrane by capillary action using 25 mM sodium pyrophosphate as the transfer solution. Start with an ~1 inch pad of the precut paper towels in a clean glass container and add transfer solution sufficient to completely saturate the towelling. Gently compress the towel pad to remove air bubbles. Place three pieces of the Whatman filter paper on the towel pad and carefully invert the gel and place it on the filter paper. Gently remove air bubbles by sliding a finger across the gel surface. Next, place the nylon membrane, labelled with a black ball-point pen, on the gel and again gently remove air bubbles. Place three pieces of the Whatman filter paper on the membrane, followed by a 2–2.5 inch stack of the paper towelling. Finally, place a glass plate on top of the stack. Add transfer solution to the container to a level just under the gel. Allow to transfer overnight.
6. Remove the membrane from the transfer stack and allow it to air-dry at RT. Place membrane between Whatman filter paper and bake *in vacuo* at 80°C for 3–5 h. Wrap with aluminium foil and store at RT.

26.3.4 DNA insert preparation

Preparation of clone insert DNA for radiolabelling is efficiently accomplished by using a polymerase chain reaction (PCR) protocol (Saiki *et al.*, 1988; Chapter 21). Most cloning vectors contain defined primer annealing sites that facilitate PCR amplification of the insert with a small amount of associated vector sequence. This procedure is extremely useful for rapidly isolating insert DNA from random λ phage cDNA clones to screen for useful RFLP polymorphisms (Severson *et al.*, 1993). Specific directions are presented for amplifying inserts from vectors with SP6 and T7 promotor

primers. Other primer combinations may require appropriate adjustment of the anneal temperature in the PCR programme.

1. Set up the PCR reaction in 0.5 mL microcentrifuge tubes as follows:
   5 μL 10× Reaction buffer.
   3 μL 25 mM $MgCl_2$.
   2 μL 5 mM dNTP mix.
   4 μL SP6 primer at ~10 ng/μL.
   4 μL T7 primer at ~10 ng/μL.
   ~1 unit *Taq* DNA polymerase.
   Template DNA for amplification use the following:
      λ phage plug preparations: add 5 μL of a 100 μL preparation.
      plasmid minipreps: add 1 ng.
      bacterial colonies or λ phage plaques: scrape the surface with a sterile toothpick and swirl the toothpick in the reaction mixture.
      ddH_2O to a final volume of 50 μL.
2. Mix gently and overlay with mineral oil.
3. Place tubes in the thermal cycler and run the following programme for 30 cycles:
   94°C for 1 min
   50°C for 1 min
   72°C for 2 min
4. When the programme is completed, remove most of the mineral oil with an Eppendorf pipette, add 50 μL chloroform, vortex, and centrifuge for 2 min.
5. Transfer the aqueous supernatant to a clean 0.5 mL microcentrifuge tube. Check a 5 μL aliquot on a 0.6% agarose gel. Store at –20°C.
6. Size-fractionate the reaction products on a 1.2% low melting temperature (LMT) agarose gel in 1× TAE running buffer.
7. Place the EtBr-stained gel on UV transilluminator and excise the slice of LMT containing the fragment of interest using a clean razor blade. Make cuts around the band as tight as possible to minimize the amount of associated LMT agarose. *Use adequate shielding and minimize skin exposure to ultraviolet light while performing this procedure as you can receive serious burns, including eye damage.*
8. Place gel slice in a 1.5 mL microcentrifuge tube and centrifuge briefly to pellet the slice. Adjust the total volume to ~400 μL with TE, pH 8.0, and incubate the tube at 65°C for 5 min. Vortex the tube briefly and immediately add 400 μL of phenol prewarmed to 37°C. Vortex well and centrifuge at high-speed for 5 min, extract the supernatant with 400 μL chloroform, and precipitate the DNA overnight at –20°C with 40 μL 4 M ammonium acetate and 1 mL 95% ethanol.
9. Centrifuge at high-speed for 10 min, decant off the supernatant and remove as much of the ethanol as possible with a 10 μL micropipette. Incubate the tube at 37°C with the cap open for ~10 min to remove remaining ethanol. Resuspend the pellet in 20–25 μL TE, pH 7.4. Estimate fragment concentration by checking a 2–4 μL aliquot on a standard agarose gel.

26.3.5 Probe radiolabelling

DNA hybridization probes can be radiolabelled with ^{32}P to very high specific activities using a simple PCR protocol (Severson and Kassner, 1995). This procedure is

complementary to PCR-based insert isolation (Section 26.3.4). Specific directions are presented for radiolabelling inserts generated with SP6 and T7 promoter primers. Inserts prepared with other primer combinations may require appropriate adjustment of the anneal temperature in the PCR programme. For insert DNA prepared by standard restriction enzyme digestion and isolation from the cloning vector, we use a completely random nine-base oligonucleotide mix (RP9) with an anneal temperature of 37°C to prime the PCR labelling reaction.

1. Place 50 ng of insert DNA into a 0.5 mL microcentrifuge tube and add ddH_2O to a final volume of 28.6 μL.
2. To set up a number of labelling reactions at one time, on ice prepare a master mix containing for each reaction: 5 μL 10× reaction buffer, 3 μL 25 mM $MgCl_2$, 4 μL SP6 primer, 4 μL T7 primer, 0.2 μL 5 mM mixture of dATP, dGTP, dTTP, 0.2 μL (~1–2 U) *Taq* DNA polymerase and 5 μL (~50 μCi) α^{32}P-dCTP. Add 21.4 μL of the master mix to each tube containing insert DNA and mix gently. *Use proper safety procedures for handling radioactive materials.* This reaction can be set up with any known primer combination for your insert. For labelling undefined inserts or inserts too long to efficiently prepare using a PCR procedure, use 9 μg of the RP9 mixture in each reaction. No mineral oil overlay is necessary.
3. Place tubes in the thermal cycler and run the following programme for one cycle:
   94°C for 2 min
   50°C for 3 min
   slow ramp up to 72°C
   72°C for 5 min
4. Prepare a small Biogel chromatography column for each probe: Plug the tip of a 1 mL Eppendorf pipette tip with a small amount of glass wool. Do not pack the glass wool too firmly. Place the tips in a holder that will maintain them in position directly over 1.5 mL microcentrifuge tubes. To set up a Biogel column in each tip, the holder can initially be held over small plastic tub. Swirl the Biogel preparation to resuspend the Biogel. Immediately add 1 mL of the Biogel to each tip. Allow the buffer to drip through, forming a gel matrix, and discard the flow-through. This will take ~15 min. Next, place a rack containing a labelled, empty 1.5 mL microcentrifuge tube directly under each tip.
5. Add 10 μL Orange G to each labelling reaction, mix, and centrifuge briefly to pellet the contents. Slowly add the reaction mixture to the surface of the Biogel matrix in the tip. Allow ~1–2 min for the mixture to penetrate the matrix.
6. Add 330 μL of Biogel elution buffer to the surface of the matrix. Collect the flow-through (containing the probe) until the Orange G dye front reaches the glass wool plug. Discard the used tip in an appropriate radioactive waste container.
7. Determine probe activity by Cerenkov counting in a liquid scintillation counter.

26.3.6 λ *Hin*dIII marker radiolabelling

λ *Hin*dIII DNA markers, included on most Southern blots, can be directly visualized on autoradiograms by including a ^{32}P-labelled λ *Hin*dIII mixture during probe hybridization. This makes RFLP allele identifications much easier to determine, particularly when comparing genotypes across more than one Southern blot. The λ *Hin*dIII DNA can be labelled with a simple fill-in reaction.

1. Digest λ phage DNA with *Hin*dIII: 50 μg λ DNA, 25 μL 10× *Hin*dIII buffer, 10 μL *Hin*dIII, ddH_2O to 250 μL total volume. Incubate at 37°C for 2–3 h.
2. Add 125 μL each of phenol and chloroform, vortex briefly, and centrifuge at high-speed for 2 min. Extract the supernatant with 250 μL chloroform, and precipitate with 625 μL 95% ethanol.
3. Centrifuge at high speed for 10 min, decant the supernatant, and wash the DNA pellet with 1 mL ice-cold 70% ethanol. Remove as much of the ethanol as possible with a 10 μL micropipette and resuspend the DNA in 400 μL of TE, pH 8.0. Store in aliquots at –20°C.
4. To prepare labelled marker: 2.5 μL 10× nick translation buffer, 2.5 μL λ *Hin*dIII DNA, 2.5 μL (~25 μCi) α^{32}P-dCTP, 1 μL Klenow, ddH_2O to 25 μL total in a 1.5 mL microcentrifuge tube. Mix gently and incubate at RT for 30 min. *Use proper safety procedures for handling and disposal of radioactive materials.*
5. Remove unincorporated ^{32}P using Biogel column chromatography (Section 26.3.5). Determine probe activity by Cerenkov counting in a liquid scintillation counter. A single λ *Hin*dIII labelling reaction can be used in hybridizations for about 3 weeks.

26.3.7 Blot hybridization and regeneration

Blot hybridizations can be efficiently performed in glass bottles in a commercial hybridization oven (Chapter 20). These instruments facilitate hybridizations with a minimum of hybridization solution and provide uniform contact of the membrane with probe DNA. Also, prehybridization, hybridization with probe, and posthybridization membrane washes can all be performed while the membrane remains in a glass bottle. This reduces the amount of handling and minimizes exposure to radioactive materials by the researcher. *Use proper safety procedures for handling radioactive materials.*

1. Prior to the initial hybridization, newly prepared Southern blots should be incubated in 200–300 mL of preblock solution with gentle shaking at RT for 3–5 h. Once preblocked, the membrane can be wrapped in Saran Wrap and stored at –20°C or you can immediately proceed with hybridization. The preblock solution can be re-used for several blots. It should be stored at 4°C and prewarmed at 65°C to redissolve the SDS prior to re-use.
2. Prewarm the hybridization oven and the hybridization solution to 65°C. Various sized glass bottles are available for use with hybridization ovens. Select a bottle appropriate for the size of your Southern blot and add hybridization solution sufficient to just cover the membrane as the bottle rotates in the oven. The same solution is used for both prehybridization and hybridization. For 300 × 35 mm bottles, we use 10–16 mL and for 150 × 35 mm bottles we use 5–7.5 mL. Return the bottles to the oven for several minutes before adding membranes. Roll the membrane with the DNA-containing side toward the inside, slide it into the bottle, and allow it to unroll against the side of the bottle. Return the bottle to the oven for 3–5 h minimum, with constant rotation. We prefer an overnight prehybridization.
3. Incubate the tubes carrying probe DNA and λ *Hin*dIII probe at 100°C for 5 min and then immediately place them on ice. Centrifuge the tubes briefly before opening them to bring the probe solution to the bottom of the tube. For

hybridizations to Southern blots for individual mosquitoes, add ^{32}P-labelled probe at ~750 000 cpm/mL of hybridization solution. For λ *Hin*dIII probe, add ~1000 cpm/mL of hybridization solution.

4. Return bottles to the oven overnight, with constant rotation.
5. Remove bottles and carefully pour the hybridization solution into an appropriate waste container. The membranes can then be washed directly in the bottles. Again, discard the wash solutions appropriately. For each wash, fill the bottle ~½ full with wash solution as follows: (i) Add wash solution I at RT and return bottle to oven for 15 min. (ii) Discard the first wash and add wash solution I prewarmed to 65°C. Return bottle to oven for 15 min. (iii) Discard the second wash and add wash solution II prewarmed to 65°C. Return bottle to oven for 15 min. (iv) Discard the third wash and add wash solution II prewarmed to 65°C. Return bottle to oven for 15 min.
6. Carefully remove the membrane from the bottle with forceps and place in a plastic tub with wash solution II at RT.
7. Trim the edges of a plastic sheet saver, leaving the folded edge intact. Place a membrane within the plastic sheet saver and press it flat to remove all bubbles and excess fluid. A rolling pin works well. Be sure that at least a 0.5 inch edge exists between the membrane and the periphery of the plastic sheet. It is important that the membrane does not dry because the probe DNA would become irreversibly bound. Wipe the exterior surface of the plastic sheet with Staticide using a Kimwipe.
8. Place the plastic sheet containing the membrane in a film cassette containing an intensifying screen. Expose the membrane to X-ray film at –80°C. Time of exposure can be estimated with practice by the intensity of signal detected with a Geiger-Mueller counter. The plastic sheets can be reused indefinitely.
9. Membranes are regenerated for additional hybridizations by incubation with gentle shaking in 0.4 N NaOH at 42°C for 30 min followed by incubation in blot neutralizer solution at 42°C for 30 min. The membranes can be wrapped in Saran Wrap and stored at –20°C between hybridizations.

NOTES

1. It is important that the DEB extraction buffer be freshly prepared. The SDS seems particularly vulnerable to precipitating out if the buffer sits for any length of time. The restriction digest may fail with DNA extracted using old buffer. It is also important that the insect tissue be thoroughly ground in the buffer before proceeding with the extraction process.
2. Acid depurination of the DNA prior to Southern blotting is not recommended because the physical degradation of purine bases can reduce the number of rounds a blot can be rehybridized.
3. Cloned inserts larger than ~2 kb are not efficiently amplified with standard PCR conditions. For larger clones, conventional plasmid miniprep, restriction enzyme digestion, and LMT agarose purification of inserts for radiolabelling is recommended.
4. Excellent positively charged nylon membranes are available from a variety of suppliers. However, there can be extreme variability in membrane performance

and some lots and portions of specific lots will fail completely. It is helpful to record the lot number for each Southern blot, because it can help with troubleshooting.

5. We prefer baking filters in preference to UV crosslinking because there is some evidence that baked filters will retain target DNA better and, subsequently, increase the number of rounds a blot can be rehybridized.

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CHAPTER TWENTY SEVEN

Microsatellite mapping of insect genomes

Liangbiao Zheng

27.1 INTRODUCTION

A microsatellite is a DNA fragment consisting of a tandemly repeated simple sequence, such as a dinucleotide or a trinucleotide (e.g. [GT]n, [GAC]n, etc.). In most eukaryotic organisms, microsatellites are dispersed throughout the genome (Hamada *et al.*, 1982). They are usually highly polymorphic in length due to a variation in the number of repeats within a given microsatellite locus as a result of uneven crossover (as in minisatellite, Jeffrey *et al.*, 1985) or slippage of the DNA polymerase during replication (Tautz, 1989). This polymorphism leads to an increased probability of finding heterozygous individuals and the microsatellite markers can be mapped by recombination since they are generally inherited in a co-dominant, Mendelian fashion (Weissenbach *et al.*, 1992).

There are several technical reasons that make the genetic mapping of microsatellites easy. First, microsatellite markers can be easily identified from a genomic library (Zheng *et al.*, 1993). An efficient way for their isolation is to clone size-selected, short (200–500 bp long) fragments of digested genomic DNA in M13 vectors. The library is then screened with an end-labelled oligonucleotide that contains the dinucleotide repeats, e.g. $[GT]_{15}$. Single-stranded DNA can be prepared from the positive plaques and the unique sequences flanking the repeats can be determined by the dideoxy termination protocol (Sanger *et al.*, 1977; Chapter 25). Second, polymorphism of microsatellites is commonly detected by the Polymerase Chain Reaction (PCR, Saiki *et al.*, 1988; Chapter 21), using primers based on the flanking unique sequences (for example, Figure 27.1). The size difference in PCR products, as a result of variation in the number of repeats, can then be detected by high resolution electrophoresis on a sequencing gel (Chapter 25). Finally, several hundred loci can be assayed from an individual insect, since minute amounts of DNA (~5 ng) are needed for each PCR reaction.

This chapter provides the basic protocol for identifying and assaying microsatellite markers as used for the construction of a recombination map for *Anopheles gambiae*. However, it can be used in this form with DNA from any other insect.

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*CCGG*CATCCGTGGATGTAGATGATCGACGATCTCAATGTGTACGTCGTCACACTACAGAGTCATATTTACAGGTTGCTAC 80

5′ ATCCCTGCTCCCCATAAAGC 3′

TCAATAGAAACTCTATTGGAGTTCGTGCAACTAGAAACGGTACTACTACACTGCATATCCTGCTCCCCATAAAGCCAG**TG** 160

TGTGCC**TGTGTGTGTGTGTGTGTGTGTGTGTGTG**TT**TGTG**TACAGTTACCAAGTAGACCTTGGAGCACGGCACAGGACCTAC 240

3′ GTTCATCTGGAACCTCGTGC 5′

GAGATCGGAGGGATTTCCCGTGCACACTTTAAATGGTTTCCCACCTTTATCAAGGTGTCGCCGCAA*CCGG* 310

Figure 27.1 An example of a microsatellite sequence (*AG2H157*) from *An. gambiae*. The *Hpa*II fragment (310 nucleotide long) identified by screening an M13 mp18 library of short DNA fragments with a single-stranded, ^{32}P-labelled $[TG]_{15}$ oligonucleotide. The sequence was determined for one strand only. The *Hpa*II sites are shown in italics, the 18 d[TG] repeats (interrupted by two C's and two T's) are shown in bold. The sequences of the forward and reverse PCR primers are shown. The expected size of the PCR product for this allele is 91 nucleotides long.

27.2 MATERIALS

27.2.1 Primers and end-labelling reagents

1. 20 μM primer (between 50–60% GC content and 18–22 nucleotides long) in double distilled H_2O (ddH_2O). Store at –20°C.
2. Phage T_4 polynucleotide kinase.
3. 10× Kinase buffer: 700 mM Tris-HCl (pH 7.6), 100 mM $MgCl_2$, 50 mM dithiothreitol. Store at –20°C.
4. γ-^{32}P-ATP (New England Nuclear or Amersham) should be stored at –20°C. Fresh and high specific activity isotope is preferred.

Caution: to work with radioactive materials, one should always wear a lab coat, goggles and gloves and work behind a Plexiglass shield. Dispose of radioactive materials properly according to the local regulations.

27.2.2 PCR

1. 5 U/μL *Taq* polymerase (Perkin-Elmer), stored at –20°C.
2. 10× PCR Buffer: 15 mM $MgCl_2$, 500 mM KCl, 100 mM Tris-HCl, pH 8.3, and 0.01% (w/v) gelatin. Store at –20°C.
3. 10 mM of each deoxyribonucleotide triphosphate (dATP, dCTP, dGTP, TTP). Store at –20°C. An ultrapure stock solution (100 mM) is available from Pharmacia.
4. Mineral oil (Sigma M-3516).
5. Microassay Falcon 3911 plate and 3913 lid (Becton-Dickinson).
6. Perkin-Elmer 9600 or MJ Research Thermal cycler PTC-100.
7. Insect genomic DNA can be prepared from fresh, frozen (at –80°C), or ethanol preserved samples (Chapter 14). The DNA is then suspended in TE (10 mM Tris, pH 7.5, 1 mM EDTA, pH 8.0) at 5 ng/μL and stored at –20°C.

27.2.3 Polyacrylamide gel electrophoresis

1. 40% polyacrylamide stock (38% polyacrylamide and 2% bis-acrylamide) in double distilled water. Store at 4°C. (*Caution: acrylamide is a potential neurotoxin; handle the powder form in a ventilated hood and wear gloves at all times.*)
2. Ultrapure urea.
3. TEMED (N,N,N',N'-tetramethylethylenediamine). Store at 4°C.
4. 10% Ammonium persulfate should be stored at 4°C and used within days. Aliquots can be stored at –20°C for several months.
5. Sequencing gel apparatus (33.5 cm wide and 42 cm long) and power supply (e.g. Gibco BRL).
6. Formamide loading dye: 98% deionized formamide, 10 mM EDTA pH 8.0, 0.025% each of xylene cyanol FF and bromophenol blue. Aliquots can be stored at –20°C for several months.
7. 3MM paper (Whatman).
8. X-ray film (Kodak XAR-5).

9. Optional equipment and materials: Savant oil pump and –80°C freezer trap; Hoefer SE1160 gel dryer; Fixing solution: 10% glacial acetic acid and 10% methanol.

27.3 METHOD

27.3.1 End-labelling reaction

Radioactive labelling of only one of the two primers is sufficient. Synthetic oligonucleotides usually have hydroxyl groups at the 5′ ends and are ready for the kinase reaction.

1. In a 1.5 mL microfuge tube, add:

3.0 μL	Primer A (20 μM)
1.5 μL	10× Kinase buffer
3.0 μL	γ-^{32}P-ATP (>5000 Ci/mMol)
1.5 μL	T4 polynucleotide kinase (10 U/μL)
6.0 μL	ddH_2O

2. Incubate at 37°C for 30 min, then heat kill the enzyme at 65°C for 10 min. Put on ice.

27.3.2 PCR amplification

1. Add 1.0 μL (~5 ng) of DNA from each individual insect to a microfuge tube. Close the cap to prevent evaporation. If many samples are to be assayed at the same time, it is much easier to use a microtitre plate.
2. Prepare a master mix (for 60 PCR reactions):

60 μL	10× PCR Buffer
12 μL	Primer A (20 μM)
15 μL	Primer B (20 μM)
3 μL	*Taq* polymerase (5 U/μL)
2 μL	dNTP mix (10 μM of each nucleotide)
423 μL	dd H_2O

3. Add the above mixture to the microfuge tube containing the labelled Primer A (15 μL). Mix well. Then dispense 9 μL of the master mix to each PCR reaction tube or well of the microtitre plate already containing 1 μL DNA template. Add a drop of mineral oil from a squeeze bottle to each tube or well of the microtitre plate. Close the cap or cover the plate with a Falcon 3913 lid. The final concentration for each component is:

Genomic DNA	0.5 ng/μL
PCR buffer	1×
Labelled primer A	0.1 μM
Unlabelled primer A	0.4 μM
Primer B	0.5 μM
dNTP (of each nucleotide)	0.2 μM
Taq polymerase	0.025 U/μL

4. Place the tubes (or plate) in the PCR machine, set to run the following programme:

Preheating:	94°C	5 min
30 cycles of:	94°C	10 s
55°C	20 s	
72°C	30 s	
Extension:	72°C	5 min
Incubate at:	4°C	(hold)

5. Keep the PCR product at 4°C in a shielded box.

27.3.3 Electrophoresis

The PCR products are heat-denatured and quickly chilled on ice. The single-stranded DNA is then separated electrophoretically using an acrylamide 'sequencing' gel. Only one strand of the PCR product will be visualized by autoradiography.

1. Add 10 μL of formamide loading dye to each PCR tube. Heat the samples at 100°C for 1.5 min (or 65°C for 10 min) and immediately chill on ice.
2. Load 2 μL of sample into each well. Run at 1500 V (or no more than 50 W) until the bromophenol blue starts running out of the gel.
3. Separate the glass plates. Blot the gel to a piece of 3MM Whatman paper. Expose to an X-ray film.
4. (Optional) While the gel is still stuck to one glass plate, fix the gel with Fixing Solution. This step should be performed in a ventilated hood. Then blot the gel to a piece of 3MM Whatman paper. Cover the top of the gel with Saran wrap and dry it using a gel dryer.
5. Develop the film.

27.3.4 Size standards

For genetic mapping of a pedigree, the most important aspect is the genotype of an individual. However, for studies of individuals from a population it may sometimes be necessary to know the exact size of each allele. The sizes of different alleles can be determined when a sequencing reaction of known sequence (ladder) is run side by side with the PCR products. Dideoxy termination sequencing with the USB kit is performed as described in Chapter 25.

1. The M13 sequencing primer (5′ d[GTAAAACGACGGCCAGT] 3′) can be labelled by the kinase reaction exactly as described above.
2. 1 μL of the labelled primer can be used directly (without purification) for annealing: 1 μL of labelled primer, 2 μL of 5× sequenase buffer (200 mM Tris-HCl, pH 7.5, 100 mM $MgCl_2$, 250 mM NaCl), and 7 μL of template DNA (0.2 μg/μL M13 vector mp18 or mp19). Incubate at 65°C for 2 min. Slowly cool down to 35°C over about half an hour. Spin briefly and keep on ice.
3. Add 1 μL of 0.1 M DTT, 2 μL of 1:5 diluted labelling, 2 μL of 1:8 diluted Sequenase. Mix and incubate at RT for 5 min.
4. Dispense 3.5 μL to each termination tube (2.5 μL of G, A, T, C), mix and incubate at 37°C for 10 min. Stop the reaction with 4 μL of formamide loading dye.
5. Heat the sample at 95°C for 1.5 min and chill on ice. Load 2 μL onto a sequencing gel.

27.3.5 Interpretation and modifications

A microsatellite allele is usually represented by several bands clustered together (Figure 27.2). This is mainly due to the slippage of *Taq* polymerase during PCR (Tautz, 1989; Weber, 1991). These artefactual bands make it difficult sometimes to differentiate between a homozygote and a heterozygote. The 'real' PCR product is usually the top (longest fragment) and strongest band. This problem could be easily resolved when a pedigree is analysed.

Apparently, some stutter bands are results of the presence of secondary structures in the PCR product, and the intensity of these bands can be reduced by using a highly denaturing polyacrylamide gel (Moore *et al.*, 1991). Genotyping using fluorescence primers instead of radioactively labelled primers has been described (Edwards *et al.*, 1991; Ziegle *et al.*, 1992). Because of the sensitivity and wide range of detection with fluorescence, artefact bands can be more easily distinguished from 'real' alleles. A second advantage is that the end-labelling reaction is no longer required. However, expensive equipment is a prerequisite for genotyping with fluorescence and this is, at the present time, restricted to few laboratories. Nonradioactive modifications of the protocol have also been developed where the PCR products are visualized by staining with ethidium bromide (Aitman *et al.*, 1991, Hearne *et al.*, 1991), or silver stain (Love *et al.*, 1990). In such cases, the PCR products can be directly separated using a native polyacrylamide gel.

NOTES

1. Sometimes synthetic oligonucleotides contain too much organic solvent which inhibits both kinase and PCR reactions. If this is the case, the oligonucleotide can easily be purified using a NAP-10 column (Pharmacia).
2. Occasionally, the plastic tubes or microtitre plates from a different batch will give erroneous results. This may be the result of the binding of *Taq* polymerase to the plastic. The addition of bovine serum albumin (BSA) or acetylated BSA (New England Biolabs) at a final concentration of 1 mg/mL could enhance the consistency of the PCR reaction.
3. If a large number of individuals are genotyped at the same time, DNA samples may evaporate before one finishes preparing all the samples. It is then better to dilute the genomic DNA to 1 ng/μL and use 5 μL increasing the total volume of each reaction to 20 μL. Adjust the amounts of all PCR reaction components accordingly.
4. A repeater pipette (Eppendorf) can be used to add the master mix to each individual reaction tube (or well). A multichannel loader, eight-channel 10 μL syringe, is available from Hamilton. Compatible combs can be obtained from Owl Scientific.
5. A premix of acrylamide solution with TBE buffer and urea can be prepared and stored at RT. However, it should be used within 2 weeks. A 40% acrylamide solution can be stored at 4°C for several months.
6. Usually one of the sequencing glass plates is siliconized (with Sigmacote, Sigma) to prevent the gel from sticking to both glass plates. The siliconized plates may be cleaned of by soaking in 10% NaOH overnight followed by extensive rinses with water (Chapter 25).
7. When blotting the gel onto Whatman paper, one should be careful not to create any space between the paper and the gel. The white paper sheet accompanying

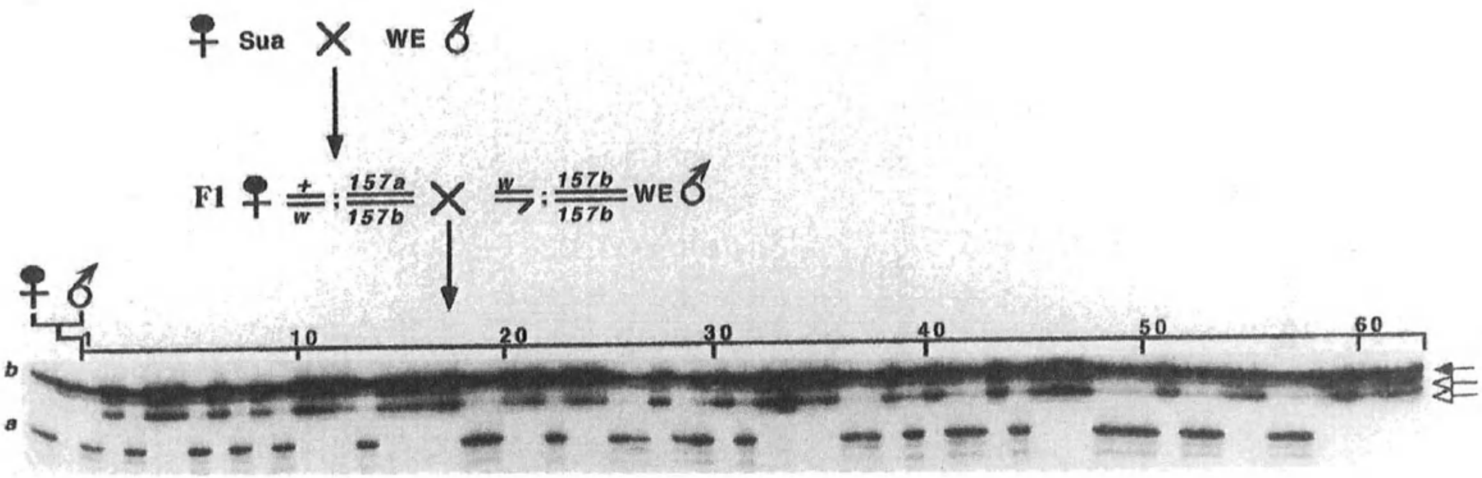


Figure 27.2 An example of primary genetic analysis of a family of *An. gambiae* mosquitoes using a microsatellite marker (*AG2H157*). PCR was performed for each of the 63 offspring and their parents as described in the text. The (+/*w*) female parent is heterozygous for the marker *AG2H157 (a/b)*, and is derived from the progeny of a cross between the Suakoko (Sua) and white-eye (WE) strains (family A in Zheng *et al.*, 1993). The WE male parent is presumed to be homozygous for *AG2H157 (b/b)*. As discussed in the text, an allele is often represented by several bands: the filled arrow indicates the real *b* allele, while the empty arrows denote artefactual products. Offspring #1–17 and #31–48 are respectively males and females with wildtype eye colour. Offspring #18–30 and #49–63 are respectively males and females with white eyes. *AG2H157*and *w* are located on different chromosomes. There are roughly equal numbers of recombinant and parental offspring: *AG2H157a –w*: 14; *a* +: 13; *b w*: 14; and *b*+: 22. Since it is known that *w* maps on chromosome X (Zheng *et al.*, 1993), the fact that male offspring can be heterozygous for two alleles of *AG2H157* indicates that this marker is autosomal and not linked to *w*.

Kodak X-ray film can be used instead of Whatman paper. However, it is not as absorbent, and one needs to be careful when using it during the blotting of the gel.

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CHAPTER TWENTY EIGHT

Polytene chromosome preparation from anopheline mosquitoes

Alessandra della Torre

28.1 INTRODUCTION

Within certain Dipteran tissues chromatids replicate repeatedly without the occurrence of any mitosis-like events. This phenomenon – known as endoreplication – leads to the production of thousands of sister chromatids within the same nucleus resulting in more extended axial parts and a number of more contracted chromomeric loops of different size. The outcome are the so-called polytene (or giant) chromosomes, which appear as bundles of extended interphase chromatids characterised by peculiar banding patterns and by an axial length approximately 100–200 times the length of the same chromosomes during mitotic metaphase. Such chromosomes can easily be observed under a compound microscope (Figure 28.1).

Although in most mosquito genera the polytene chromosomes are either not suitable for cytological manipulation or only partially replicated, excellent preparations can be obtained from many *Anopheles* species. Adequate polytene chromosome squash preparations can be made from the salivary glands of late fourth instar larvae and from the nurse cells of half-gravid adult females; a lower degree of polytenization also occurs in other tissues. *Anopheles* species are characterised by one pair of heteromorphic sex chromosomes (X and Y) and two pairs of autosomes (numbered 2 and 3). However, since the heterochromatic region of the X chromosome and the whole Y chromosome are under-replicated, the typical anopheline polytene complement is substantially constituted by the euchromatic region of the X-chromosome and by the two metacentric or submetacentric autosomes. This means a total configuration made of five polytenic arms (X, 2R, 2L, 3R, 3L).

Photomicrographs and drawings of the whole polytene complement of any given species have lead to the construction of physical maps, which may be integrated with genetical maps through *in situ* hybridization (Chapter 29), thus allowing the localization of genes or other DNA segments. Moreover, chromosomal variations such as the rearrangements of the banding pattern due to paracentric inversions can be utilized as genetic markers in mapping procedures. For a general review on polytene chromosomes in genetic research, see Sorsa (1988).

Molecular Biology of Insect Disease Vectors: A methods manual. Edited by J.M. Crampton, C.B. Beard and C. Louis. Published in 1997 by Chapman & Hall. ISBN 0 412 73660 8.



Figure 28.1 Nurse cell polytene chromosomes of *Anopheles gambiae.*

Analysis of polytenic complement homologies has proved to be of great value in elucidating inter-relationships between species, even in the case of well differentiated taxa, such as those within the subgenus *Cellia* (Coluzzi *et al.*, 1979; Green, 1982). Paracentric inversions also represent unique diagnostic tools in distinguishing members of sibling species complexes: when dealing with field-collected specimens, sibling species may, in most cases, be recognized as carriers of alternative arrangements – one or more inversions – coexisting in an apparently panmictic sample in the absence of significant deficit of the expected heterozygotes. Following the first identification of sibling species in the *An. maculipennis* complex (Frizzi, 1947, 1953; Kitzmiller *et al.*, 1967), in the last 30 years cryptic taxa have been chromosomally characterized within various *Anopheles* groups, some of which are of great relevance in malaria epidemiology, such as *An. gambiae* (Coluzzi, 1966; White, 1973; Davidson and Hunt, 1973; Coluzzi *et al.*, 1979, 1985), *An. farauti* (Bryan and Coluzzi, 1971; Mahon and Miethke, 1982; Mahon, 1983), *An. culicifaces* (Green and Miles, 1980; Subbarao *et al.*, 1983), *An. maculatus* (Green *et al.*, 1985).

In addition to fixed inversions, polymorphic inversions are also frequently observed in anopheline species and constitute a useful tool for vector analysis at the intraspecific level. Paracentric inversions can be considered as mechanisms that preserve, through the suppression of crossing-over in the heterokaryotypes, gene associations arising in temporary isolates subject to flush and crush in geographically and/or ecologically marginal zones (Coluzzi, 1982). Thus, carriers of alternative arrangements in a vector population might express ecotypic divergences and epidemiologically important heterogeneities. This phenomenon has been extensively studied in *An. gambiae* s.s. and in *An. arabiensis*, whose capacity to associate with man

in a wide range of ecological conditions appears to be related to their remarkably high degree of inversion polymorphism. Moreover, evidence from field and laboratory investigations suggests that chromosomal polymorphisms may be an important source of variation that influences the efficiency of a species as vector of human plasmodia (Coluzzi *et al.*, 1979, 1985; Bryan *et al.*, 1987; Petrarca and Beier, 1992).

The following sections will describe the technique to be used to obtain chromosomal preparations from *Anopheles* spp. Section 28.3 describes the standard procedure to prepare slides from nurse cells of fresh half-gravid females. The Notes describe how to identify the correct life stages required for cytogenetic preparations and the modifications to the standard technique to be applied when preparing chromosomes from Carnoy-fixed half-gravid specimens, or from salivary glands of fourth instar larvae. The above mentioned protocols are partially derived from those described by French *et al.* (1962), Coluzzi (1968), Hunt (1973) and White *et al.* (1975); however, they are mainly the result of the experience acquired by the staff of the Istituto di Parassitologia 'La Sapienza' (Italy), where more than 150 000 *Anopheles* specimens have been chromosomally characterized in the last 25 years.

28.2 MATERIALS

1. Modified Carnoy's fixative (three parts of pure ethanol and one part of glacial acetic acid), to be used when the dissections are not performed on fresh specimens. This fixative can be stored for months at RT.
2. Anaesthetic (ethyl ether, CO_2 or freezing facilities).
3. 5% and 50% aqueous solution of propionic acid.
4. Pure lactic acid.
5. Aceto-lactic orcein stain: 2% by weight of synthetic orcein powder diluted in 1 part glacial acetic acid and 1 part pure lactic acid. Add the powder very slowly to the solution mixing with a magnetic stirrer. Filter the solution through a Whatman 3MM paper. Store indefinitely at RT.
6. Absorbent paper.
7. Grease-free and dust-free microscope slides.
8. Grease-free and dust-free 22 mm^2 coverslips (thickness 0.15–0.20 mm)
9. Dissecting needles.
10. Dissection microscope.
11. Nail varnish for semi-permanent mounting.
12. Phase-contrast compound microscope with magnification up to 400×–1000×.
13. For permanent mounting the following additional tools are required: siliconized 22 mm^2 coverslips, a pair of forceps, a razor blade, liquid nitrogen, ethanol and mounting media, such as Phenol-Balsam or DPX Mountant for histology (Fluka, Code No.44581) or other analogous chemicals. The siliconization procedure should be carried out under a fume hood, as follows: dip a grease-free and dust-free coverslip in a siliconizing fluid – such as Repel-Silane (Pharmacia, Code No.80-1129-42; LKB, Code No.1850-252) or analogous chemicals from other companies – for a few seconds, wash in distilled water and air-dry. Clean with tissue paper before use; siliconized coverlips can be cleaned and used repeatedly, taking into account that silicon wears off eventually.

28.3 METHODS

1. Choose a half-gravid female *Anopheles* from your live samples (Section 28.4.1)
2. Anaesthetize the specimen by exposing it to ethyl ether, CO_2, or by freezing it at −20°C for 30–60 s. When processing several specimens, it is possible to anaesthetize and keep them on a humid surface after removing the head. This will keep the mosquitoes from flying away, while remaining alive and suitable for cytogenetic preparation for at least 30 min (polytene chromosomes from a dead specimen degenerate very fast).
3. Remove legs and wings and place the mosquito on a slide.
4. Pierce the thorax laterally with a dissection needle.
5. Pull off the first three abdominal segments directly in a drop of 5% propionic acid; the ovaries will generally be extracted simultaneously (Figure 28.2c).
6. Isolate the ovaries (Figure 28.2d).
7. Transfer the ovaries in a drop of 50% propionic acid onto a grease-free and dust-free slide.
8. Leave the ovaries in the solution until they have swollen to approximately twice their original size, i.e. 2–5 min. Avoid drying the tissues.
9. Divide the two ovaries laterally and separate the follicles with the needles. Each ovary can be utilized for a separate preparation.

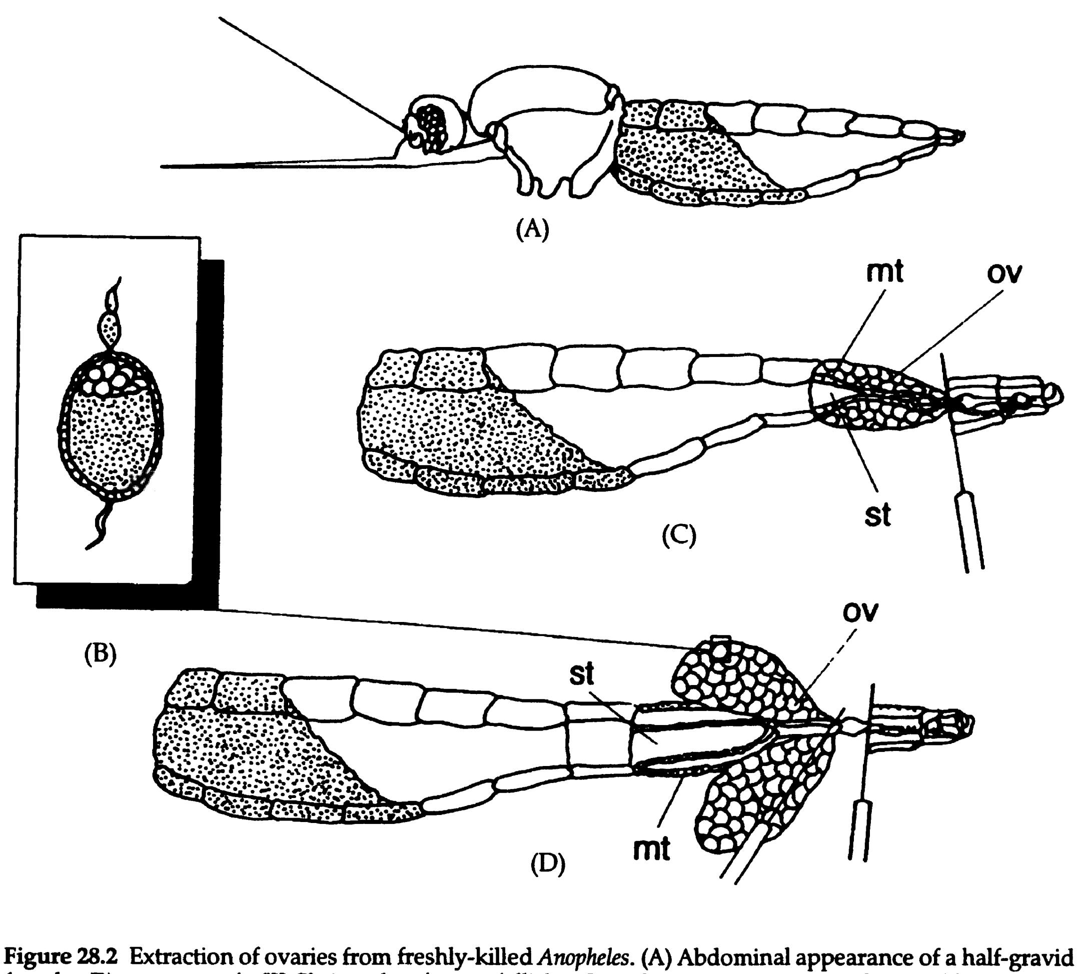


Figure 28.2 Extraction of ovaries from freshly-killed *Anopheles*. (A) Abdominal appearance of a half-gravid female; (B) structure of a III Christophers' stage follicle; (C) and (D) extraction procedures. Abbreviations: ov, half-gravid ovary; mt, Malpighian tubules; g, gut

10. Add a drop of 2% lacto-aceto orcein to the drop of propionic acid with the follicles and stir with the needle to ensure homogenous staining.
11. Stain the tissue for 1–5 min.
12. Draw off the excess stain with absorbent paper and wash the tissue with several changes of 50% propionic acid. After washing, the follicles should be have a pale pink colour.
13. Place a grease-free and dust-free 22 mm^2 coverslip on the tissues.
14. Squash the preparation by tapping gently on the coverslip with the back of either a needle or a pen, or with a wooden stick, in order to break the follicles and to spread the chromosomes. Avoid large movements of the coverslips on the slide by holding the corner with a fingertip lined with filter paper. Since propionic acid acts as a protecting cushion, a limited amount of movement does not harm the preparation and will help the spreading of the chromosomes. Care should be taken not to use too much liquid or some material may be lost: note that some of the best spread chromosomes often lie at the edges of the coverslip.
15. Beginners should check at this stage the degree of spreading of the chromosomes under a compound microscope at 100× magnification and, if necessary, repeat step 14.
16. Wrap the slide between layers of blotting paper and press firmly with the palm of the hand to help flattening of the chromosomes. Excess stain will be taken up by the paper. In this phase be very careful to avoid even the slightest movement of the coverslip, which would seriously damage the preparation.
17. The slide is now ready for microscopical examination under a compound microscope. Polytene chromosomes may be scanned at 100× or 250× magnification and banding patterns are readable at 400x. For a detailed examination 1000× magnification is recommended.
18. Unsealed preparations are readable for a few days if stored at RT, for longer if stored at 4°C and for months if stored at –20°C. To prevent infiltration of air under the coverslips, it is possible to place small amounts of lactic acid on their sides. However, caution should be taken in limiting this procedure, due to the decolorizing action of this substance.

NOTES

Correct mosquito stages for polytene chromosome analysis

The most crucial step to obtain excellent polytene chromosome preparations is to choose the mosquito specimen at the correct stage of ovarian development – the half-gravid stage (Figure 28.2a) – or at the correct larval stage, corresponding to the early–mid fourth instar larva.

In blood-fed females the polytenization process occurs in the nurse cells during the gonotrophic cycle and it is known to be linked to a high level of mRNA transcription due to the massive production of yolk. Polytenization already begins in the nurse cells of pregravid females, i.e. at Christophers' late II stage, but reaches its top at Christophers' III stage (Figure 28.2b). As egg development proceeds and the yolk fills most of the follicle, nurse cells begin to degenerate and polytene chromosomes disappear after Christophers' early IV stage. Half-gravid females may be recognized by their abdominal appearance, when the area occupied by the developing ovaries goes from the 6th dorsal to the 4th ventral abdominal segment (Figure 28.2a). Under average tropical conditions (26–28°C and 60–70% RH) this stage is reached 16–18

hours after the blood meal, while the time-interval becomes longer as the temperature decreases. It should be noted that most anophelines require two consecutive blood meals to start and complete the first gonotrophic cycle. To obtain a more synchronous egg maturation under laboratory conditions, it is therefore better to blood-feed females which have completed at least one gonotrophic cycle.

Late III or early IV instar larvae from the field or from healthy laboratory colonies are suitable for good salivary chromosome preparations, although the degree of polytenization is not optimal before the third moult. The presence of prepupal trumpets within the thorax indicates that the specimen is too old and that the polytene chromosomes have already begun to degenerate.

It is important to note that, although the banding patterns from the two tissues are essentially homologous, most bands are not easy to homologize because their expression, i.e. state of puffing, does not correspond.

Collection and preservation of adult specimens from the field

The method described above implies the use of freshly dissected specimens, i.e. the possibility for the laboratory either to rear *Anopheles* mosquitoes, or to collect them directly in the field. However, when dealing with tropical species, the supply of live mosquitoes is often difficult. Therefore a method to fix the females at the half-gravid stage and to preserve them for long period has been developed. The method is described here, together with the corresponding changes to the standard procedure:

1. Drop whole half-gravid specimens in Carnoy's fixative alive or after soft anaesthesia. If possible use freezing or CO_2 and avoid anaesthetizing with chemical agents, as these may have a detrimental effect on the nuclei containing polytene chromosomes. Live females collected at the pregravid stage should be kept at the right climatic conditions until they reach the correct stage of ovarian development. Half-gravid females knocked out by pyrethrum spray catches are suitable if fixed immediately. Do not exceed 100 specimens per 25 mL of Carnoy's fixative. If only ovaries are to be fixed, dissection must be performed without using any saline solution and ovaries must be immediately transferred to numbered microvials containing the fixative; all other mosquito body-parts should be numbered accordingly.
2. Make sure you provide each field sample with the following data: date of collection; description of the collecting site; type of collection; any other relevant information.
3. Mosquitoes should be kept in the fixative at least 24 h at RT before being used for chromosome preparations. The vials can be stored for 1–2 months at 4°C and for years at –20°C. Avoid multiple thermal changes.
4. For chromosome preparations separate the abdominal segments containing the ovaries from the rest of the body, isolate the ovaries on a grease-free and dust-free slide and add a drop of 50% propionic acid.
5. Continue the standard procedure from step 28.3.8, taking into account that the longer the fixation in Carnoy's, the harder the follicles become. The strength of the tapping on the coverslips must be adjusted to the storage length in order to obtain a comparable degree of chromosome spreading.

Chromosome preparations from larval salivary glands

Although good preparations can be obtained from larvae preserved in Carnoy's, dissection is in this case very difficult and often unsuccessful. The following procedure describes how to obtain good chromosome preparations from freshly-killed larvae:

1. Choose a larva at the correct stage of development (see above).
2. Collect the larva with a pipette, rinse it in distilled water and drop it onto a piece of clean, dry filter paper to remove the liquid. Transfer it to a drop of 5% propionic acid on a clean slide.
3. Cut off and discard the abdomen. The salivary glands lie antero-laterally in the thorax.
4. Insert one needle from the thorax rear and run the other needle over it to sever the layers between and open it completely along the mid-dorsal line.
5. Gently pull the head to extract the glands, which often remain attached to the head by the salivary ducts. Each salivary gland consists of two distinct lobes: a spherical part containing cells with excellent chromosomes, and a more pliant bag of cells with smaller nuclei and chromosomes.
6. Pick up the glands and transfer them on a drop of 50% propionic acid on a grease-free and dust-free slide.
7. Continue the standard procedure from step 28.3.8. Note that salivary gland cells which contain polytene chromosomes are fewer and softer than nurse cells. It is therefore crucial to avoid loss of material and to tap very gently on the coverslip in order to preserve chromosome integrity.

Permanent mounting

If permanent mounting is not required, an application of nail varnish on the sides of the coverslips is sufficient to keep the chromosome preparations – which are usually kept at 4°C – usable for over a year, though some drying out does inevitably occur. However, for extremely important preparations permanent slides can be obtained as follows (Conger and Fairchild, 1953):

1. Apply the standard procedure, using siliconized coverslips.
2. After step 28.3.16, dip the slide in liquid nitrogen for a few seconds.
3. Snap off the coverslip with a razor blade. The chromosomes should now stick to the nonsiliconized slide.
4. Dip the slides immediately in 95% ethanol and leave for 5 min; transfer to 100% ethanol for 5 min.
5. Remove and cover the tissues immediately with a drop of mounting medium.
6. Dip a clean coverslip in 100% ethanol, remove the excess ethanol by gently touching one edge with filter paper, and lower over the preparation.
7. Dry overnight for the mounting medium to permeate under the coverslip. Placing a uniform weight (about 50 g) over the slides helps to maintain chromosomal spreading and facilitates drying. Keep the slides flat for a few days, until drying is completed.

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CHAPTER TWENTY NINE

In situ hybridization to Anopheles polytene chromosomes

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29.1 INTRODUCTION

Many medically important insects, particularly in the order Diptera, have tissues with readily accessible, giant polytene chromosomes that can be used to physically map fragments of DNA or RNA. The technique of polytene chromosome *in situ* hybridization was developed and first used with *Drosophila* chromosomes (Gall and Pardue, 1969; Atherton and Gall, 1972), but it has since been used effectively with a variety of other Diptera, including chironomids, sciarids, several cyclorraphan flies, and anopheline mosquitoes (e.g., Bedo and Howells, 1987; Schmidt *et al*, 1988; Graziosi *et al.*, 1990; Zacharopoulou *et al.*, 1992; Kumar and Collins, 1994). DNA probes have been mapped by *in situ* hybridization to the polytene chromosomes of a number of *Anopheles* species using fluorescence *in situ* hybridization (FISH) (Marchi and Pili, 1994) and by using biotin-labelled DNA probes (e.g. Graziosi *et al.*, 1990; Kumar and Collins, 1994; Salazar *et al.*, 1994; Besansky *et al.*, 1994).

The protocol described in this chapter is based on the technique adapted by Kumar and Collins (1994) specifically for the ovarian nurse cell polytene chromosomes of the mosquito *Anopheles gambiae* and its sibling species. DNA probes are labelled by nick translation with the biotin-nucleotide analogue biotin-14-dATP, and the hybridized probe is detected by sequentially incubating the chromosomes with alkaline phosphatase covalently bound to streptavidin and then the dye substrates NBT and BCIP. This technique is sufficiently sensitive to detect labelled DNA fragments as short as 100 bp and versatile enough to be used with either ovarian nurse cell or salivary gland polytene chromosomes from probably any *Anopheles* species with morphologically well defined polytene chromosomes. Another useful attribute of this technique is that several DNA fragments can be hybridized simultaneously (horizontal hybridization), or a slide can be successively re-probed (vertical hybridization) with four or more labelled probes without significant loss of chromosome morphology.

Most of the reagents used in this procedure are available in kit form from GIBCO BRL (Life technologies, Gaithersburg, MD, USA), and the procedure is only slightly modified from the protocols that come with the GIBCO BRL kits, Bionick Labelling

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System and *In situ* Hybridization and Detection kits. The modifications associated with this protocol have been developed to optimize chromosome morphology while producing a strong, but specific, hybridization signal to *Anopheles* polytene chromosomes. Chromosome preparation is based on procedures described by Green and Hunt (1980) (Chapter 28).

29.2 MATERIALS

29.2.1 Preparation of chromosome squashes

1. Modified Carnoy's solution: 1 part glacial acetic acid: 3 parts absolute ethanol. Prepare just before use.
2. 50% Propionic acid in H_2O.
3. Siliconized coverslip. Various siliconizing agents can be used. A nontoxic agent (Gel slick™, J.T. Baker, Phillipsburg, USA) works well provided the coverslips are left in the agent overnight.
4. 50%, 70%, 90%, 100% ethanol solutions: v/v H_2O.

29.2.2 Probe preparation

1. GIBCO BRL Bionick™ Labelling System: catalogue No.8247SA (Life Technologies). The kit contains the following solutions, all of which can be purchased either in kit form or prepared individually:
   a. 10× dNTP mix: 0.2 mM each dCTP,dGTP, dTTP
      0.1 mM dATP
      0.1 mM Biotin-14-dATP
      500 mM Tris-HCl (pH 7.8)
      50 mM MgCl2
      100 mM β-Mercaptoethanol
      100 μg/mL Nuclease-free Bovine serum albumin
   b. 10× Enzyme mix: 0.5 U/μL Polymerase I
      0.0075 U/μL DNase I
      50 mM Tris-HCl (pH 7.5)
      5 mM Magnesium acetate
      1 mM β-Mercaptoethanol
      0.1 mM Phenylmethylsulfonyl fluoride
      50% (v/v) Glycerol
      100 μg/mL Nuclease-free Bovine serum albumin
   c. Stop buffer 300 mM EDTA.
   d. Distilled water.
2. 20×SSC: 3 M NaCl, 300 mM sodium citrate, pH 7.0.
3. 1 M sodium phosphate: 1 M sodium phosphate Monobasic, 1 M sodium phosphate dibasic, pH 6.5.
4. 50× Denhardt's solution: 5 g Ficoll (Type 400, Pharmacia), 5 g Polyvinylpyrrolidone, 5 g Bovine serum albumin (Fraction V, Sigma), 500 mL H_2O (Sambrook *et al.*, 1989). Filter sterilize, aliquot and store at –20°C.

29.2.3 Prehybridization

1. Glacial acetic acid: methanol: 1:3 v/v.
2. 1× PBS: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 (anhydrous), 1.5 mM KH_2PO_4, pH 7.2. Filter through 20 µm Nalgene filter.
3. 4% Paraformaldehyde: w/v in 1× PBS. Stir at 65°C until solids have dissolved, cool to RT and filter sterilize through 0.45 µm filter. Prepare fresh weekly and store between 0-4°C. *Note that paraformaldehyde is harmful if inhaled or comes in contact with the skin.*
4. 50%, 70%, 90%, 100% ethanol solutions: v/v in H_2O.

29.2.4 Hybridization

1. Gibco BRL *in situ* hybridization and detection system: catalogue no: 18250-019 (Life Technologies). The kit contains all the following solutions (in 2.4–2.5) except for the rubber cement, SSC, TBS and ASB. All the reagents and solutions can be purchased either in kit form or prepared individually.
2. 20% Dextran sulfate: w/v in formamide. Deionize the formamide by stirring 5 g/100 mL of AG501-X8 (D) 20–50 mesh resin (catalogue No: 143-6425, Bio-Rad Laboratories, Richmond, CA USA) to remove salt impurities and hydrolysis products. After removal of resin, pass the formamide twice through Whatman 1 filter paper (Whatman Laboratory Division, Kent, England) (Sambrook *et al.*, 1989). Add the dextran sulfate to the formamide and allow it to dissolve for 2 days. Agitate occasionally to speed up dissolving process. *Formamide is a teratogen and so wear gloves when handling.*
3. Carter's Rubber cement (Item no: 08-450, Avery Dennison, Framingham, MA, USA or any rubber cement).

29.2.5 Detection

1. Blocking solution: 50 mg/mL protein in 100 mM Tris-HCL (pH 7.8), 150 mM NaCl, 0.2 mg/mL sodium azide. *Sodium azide is toxic so handle with care.*
2. Conjugate dilution buffer: 100 mM Tris-HCl, 150 mM $MgCl_2$, 10 mg/mL Bovine serum albumin Fraction V, 0.2 mg/mL sodium azide.
3. Streptavidin–alkaline phosphatase: 40 µg/mL in conjugate dilution buffer.
4. TBS: 100 mM Tris base, 150 mM NaCl. Adjust pH to 7.5 with 1 N HCl.
5. ASB: 100 mM Tris base, 150 mM NaCl, 50 mM magnesium chloride ($MgCl_2.6H_2O$).
6. Nitro-blue-tetrozolium (NBT): 75 mg/mL v/v in 70% dimethylformamide.
7. Bromo-chloro-indolyl-phosphate (BCIP): 50 mg/mL in 100% dimethylformamide.

29.3 METHOD

29.3.1 Probe preparation

1. Label probes according to the protocol in the Gibco-BRL Bionick labelling kit (described below).

2. Prepare 50 μL labelling reaction mixture in 1.5 mL microfuge tubes: 5 μL 10× dNTP mix, 5 μL 10× Enzyme mix, DNA, and water to 50 μL. We use between 100 ng and 1 μg of DNA (exclusive of vector, in the case of cloned DNA), depending on the length of the probe and whether or not it is used with a homologous or heterologous species. Smaller amounts typically give less non-specific staining. Vector sequences are usually not removed from the probe.
3. Centrifuge for 5 s at 15 000 g.
4. Incubate at 16°C for 1 h.
5. Add 5 μL stop buffer.
6. Precipitate DNA by adding 5 μL of 3 M sodium acetate, mix well, add 100 μL of cold 100% (–20°C) ethanol. Invert the tube several times to ensure even mix, freeze at –70°C (dry ice) for 15 min or at –20°C for 2 h, centrifuge at 15 000 g for 10 min, discard supernatant, air or vacuum dry.
7. Dissolve DNA in 50 μl of dH_2O.
8. Repeat step 6.
9. Resuspend DNA in 800 μL 2× hybridization buffer. We estimate 20% loss of DNA in the double precipitation process of the DNA, thus resuspension in 800 μL of 2× hybridization buffer should give a final concentration of 1 μg/mL to 0.1 μg/mL, depending on whether the amount of probe labelled was 1 μg or 100 ng.
10. To save time, horizontal hybridization can be conducted where many probes could be used simultaneously on a single slide to detect and locate all at the same time. This can be done in two ways:
    a. Biotin-label more than one probe, by including different DNA samples into the same 50 μL biotin labelling reaction. Ensure that the total concentration of DNA does not exceed 1 μg in the reaction mixture. Resuspend DNA in 800 μL of 2× hybridization, as you would for a single DNA probe.
    b. Label each DNA probe separately in each 50 μL biotin labelling reaction. Depending on the number of individual probes to be used, resuspend each at the appropriate concentration of hybridization buffer, such that the final DNA concentration on the slide will be 100 ng per probe and the final concentration of hybridization buffer will be 2x. We have successfully used as many as six probes simultaneously.
11. Store probes at –80°C for long term storage. The biotinylated probes will have a shorter lifespan if stored at –20°C.
12. This protocol has been modified from that recommended in the GIBCO BRL *in situ* hybridization kit and so some reagents will run out before others in the hybridization kit.

29.3.2 Chromosome preparation

1. The general method used to produce chromosome squashes is discussed in Chapter 28, but chromosome preparation for *in situ* hybridization requires somewhat more care. Preparation of completely flat chromosomes is of paramount importance to obtain good *in situ*s. Various people use different techniques to prepare and flatten their chromosome and this is very much a matter of experience. Items such as eraser ends of pencils and mattress needles are just two of the more common instruments used to tap preparations. Firm vertical thumb pressing is recommended too, as this very often provides enough force to sufficiently flatten the chromosomes.

2. The squashes must be overlaid with a siliconized coverslip as the coverslip has to be removed to dehydrate the chromosomes.
3. Once the slides have been sufficiently tapped and pressed, place the slide on a slide warmer at 60°C for 1 min to allow the chromosomes to stick to the slides.
4. Place the slides in a humid chamber in a refrigerator (0–4°C) for 12 h to further flatten the chromosomes.
5. Dip the slides in liquid nitrogen and wait until bubbling stops before prising the coverslip off from one corner with a scalpel blade.
6. Immediately place the slides in -20°C 50% ethanol and leave at 4°C for 2 h.
7. Dehydrate the chromosomes further by placing the slides in an ethanol series of 70%, 90% and 100% for 5 min each at 4°C.
8. Air-dry the slide and place in a slide box and keep at RT until needed.

29.3.3 Prehybridization

1. Slides that are more than 4 weeks old need to be refixed in 1:3 glacial acetic acid: methanol solution at RT for 10 min and air-dried, dehydrated in 100% ethanol and air-dried again.
2. Immerse slides in 1× PBS for 20 min at RT.
3. Fix slides in 4% paraformaldehyde for no more than 1.5 min.
4. Dehydrate the slides through an ethanol series of 50%, 70%, 90%, 2× 100% for 5 min each at RT.
5. Air-dry the slides.

29.3.4 Hybridization

1. Probe each slide with a mixture of 35 μL of biotinylated probe DNA and 35 μL of 20% dextran sulfate to give a final probe working concentration of between 0.05 μg/mL and 0.5 μg/mL. Mix the biotinylated DNA probe thoroughly with the 20% dextran sulfate making sure to prevent air bubbles before dropping on to the chromosome spreads. If air bubbles form, spin at a low speed to remove them. Also warming the slides at 90°C for 5–10 s before adding the probe prevents the formation of air bubbles under the coverslip.
2. If a slide is to be hybridized with several probes, each individual probe should be labelled at a concentration such that the final concentration of the combined probes will be at least 100 ng per individual probe and no more than 1 μg total DNA concentration.
3. Place a coverslip onto the hybridization mixture to spread over the entire chromosome preparation. Avoid trapping air bubbles between coverslip and chromosome spreads.
4. Heat slide at 90°C for 12 min to denature the probe and target DNA simultaneously.
5. Seal edges of coverslip with rubber cement.
6. Place slides in a humid chamber and hybridize for 4–12 h at desired hybridization temperature. Good hybridization signals are generally obtained at hybridization temperatures of 42°C for homologous probes and temperatures as low as 37°C for heterologous probes. Hybridization above 42°C destroys chromosome morphology and hybridizations below 37°C increases background signals.

29.3.5 Posthybridization

1. Roll rubber cement from edges of coverslip. Avoid moving the coverslip while removing the rubber cement as this may scratch the chromosome spreads. Remove coverslip by washing the slides in 0.2× SSC.
2. Rinse the slides in two washes of 0.2× SSC for 20 min in each, the first at hybridization temperature (prewarm 0.2× SSC) and the second at RT. The concentration of the SSC can be adjusted from 0.1× SSC to 2× SSC, depending on the stringency of the washes required.

29.3.6 Detection

1. Cover each slide with 250 μL of blocking solution and incubate in a humid chamber for 15 min at hybridization temperature.
2. Meanwhile prepare a working conjugate solution by mixing 10 μL of streptavidin-alkaline phosphatase with 90 μL of conjugate dilution buffer for each slide.
3. Wick away the blocking solution and drop the working conjugate solution over the spreads. Incubate in a humid chamber for 15 min at RT.
4. Immediately immerse each slide in TBS for 15 min at hybridization temperature. Wash slides again in TBS at RT. Do not let the slides dry after adding conjugate.
5. Wash each slide in ASB at RT for 10 min.
6. Meanwhile prepare the NBT/BCIP stain by adding 200 μL of NBT and 166 μL of BCIP to 50 mL of ASB prewarmed to 37°C. The above mixture of NBT/BCIP applies to ovarian nurse cell chromosome squashes only. Use a lower NBT/BCIP concentration of 50 μL NBT and 41.5 μL BCIP per 50 mL of ASB for salivary gland chromosomes. Salivary gland chromosomes have a higher propensity for absorbing the stains and using the higher NBT/BCIP concentration will result in the entire chromosomes appearing violet in colour.
7. Incubate the slides at 37°C. Periodically check the slides (half-hour intervals) until desired level of signal is achieved. For periodic microscope viewing cover, the chromosome spread and residual staining solution with a coverslip. The development of a blue colour under phase contrast and a purple-violet colour under bright field indicates site/s of hybridization. It often happens that some chromosome bands refract light in such a way that they appear blue under phase contrast microscopy, but only bands with hybridization signals will appear purple-violet in colour under bright field. Slides have been incubated in the staining solution for as short a time as 1 h for large homologous probes and up to 12 h for heterologous probes.
8. Once the desired level of signal has been achieved wash the slides with distilled H_2O to stop colour development, air-dry and store at RT in slide boxes. We have not seen any visible loss of signal in slides of more than 2 years old. Each time the slides need to be viewed under the microscope, or if photographs need to be taken, cover the spreads with a drop of distilled H_2O and a clean coverslip. When viewing has been completed float the coverslip off with excess water, air-dry and place back in slide box. Ensure immersion oil does not contact the chromosome spreads. If there is some contact, then wash the slide briefly with 100% ethanol and immediately rinse with distilled H_2O.

9. After a slide has been used to locate sites of hybridization of one probe, it can be re-hybridized with another probe with a different sequence. Use the same procedure as described above starting from the prehybridization 1× PBS wash (Section 29.3.3, step 2). A slide can be reused in this vertical hybridization fashion six or more times, with little loss in chromosome morphology and previous hybridization signals, and without significant development of background staining.

NOTES

1. For anophelines, we routinely use no more than half the number of ovules from one ovary per slide. Because the number of polytene nuclei in salivary glands is much lower than in ovaries, we typically use 2–3 pairs of salivary glands per spread. Using more material increases the amount of extraneous tissue, which severely compromises the number of adequately flat chromosomes that will be obtained. Removal of the outer calyx of each ovule in the case of ovarian nurse cell preparations, trachea, any connecting tissue and mosquito scales, considerably improves the quality of the spreads.
2. Label individual slides with a pencil or scratch the labels with a diamond tip pencil. Ink labels will run over slides in the hybridization process.
3. Since flat chromosome squashes are of such importance, we suggest that the slides be viewed dry without a coverslip under a microscope after the first dehydration process. Chromosomes with regions of refractile appearance (mirror-like shine) are unsuitable.
4. The edges of the chromosome preparations originally demarcated by the coverslip edge are sometimes hard to see and become especially difficult to see after the PBS and other washes. Scratching the slide close to the edges of the chromosome spreads with a diamond tip pencil after the first dehydration process alleviates this problem and also helps to retain the blocking and working conjugate solutions over the spreads.
   Do not scratch the slides before immersing in liquid nitrogen as the slides will then often break during this process.
5. This technique has worked when entire plasmid or bacterial artificial chromosome (BAC) clones containing inserts ranging from 100 bp to 100+ kb in size have been biotin labelled and used as probes, although the signals of small inserts (<150 bp) are weak and take longer to develop. For small inserts (<150 bp), improved signal to background is obtained by excising and labelling only the insert. Slides hybridized with short sequences should also be left in the staining solution for many hours, as small DNA probes probably have a lower specific activity due to the labelling process used. Re-hybridizing the same slide with the same probe a second time can improve the strength of the signal. This is particularly true when dealing with short DNA probes.
6. Clean DNA is required for good quality *in situ*s as this will significantly reduce background nonspecificity and will maintain good chromosome morphology. This applies to probes of all sizes. The Magic Minipreps DNA-binding resin system of Promega (Promega Corporation, Madison, WI, USA) produces suitably clean DNA.

7. The technique has also proved successful in locating simple tandem dinucleotide-containing sequences to *Anopheles gambiae* chromosomes. Since the simple repeat-containing part of the probe may be represented in many different parts of the genome, such probes can produce high levels of nonspecific staining. We recommend carefully following the development of the signal, since the primary signal is the first to appear and is often rapidly followed by emerging secondary, nonspecific signals.
8. No signal anywhere, high level of staining everywhere with no indication of a specific signal, and poor chromosome morphology, are three of the most common problems. Nonspecific background and poor chromosomal morphology (no banding pattern) very often occur together, and this problem can often be solved by reducing the working concentration of probe DNA. Increasing the hybridization stringency by increasing hybridization temperatures and posthybridization washes may also help to reduce background staining. No signal is very often overcome by increasing the working concentration of probe DNA, or by lowering the hybridization temperature and decreasing the stringency of the posthybridization washes. If chromosomes appear puffed or ragged, this is usually due to over-denaturation of the target DNA and new slides should be denatured at a lower temperature. There is a narrow window at which optimal denaturation of the chromosomal DNA occurs (Leitch *et al.*, 1994) and so far we have had no problems at a denaturation temperature at exactly 90°C.
9. In the case of salivary gland chromosomes, lowering the PBS and TBS washing solutions to 0.1× solutions helps maintain clarity of bands.
10. The hybridization and detection procedure requires 1.5 days with an overnight hybridization included. From dissection of polytene chromosome containing tissues to production of dehydrated spreads requires about 1.5–2 days.
11. The biotin-labelled probe DNA using the biotin-streptavidin detection system has some advantages in that it is nonradioactive, produces a stable signal, does not require fluorescent microsocope facilities and pictures can be produced in black and white. One disadvantage of the Biotin-nick translation method for labelling probes is that it does not work efficiently for probes less than 100 bp. However, nucleic acids less than 100 bp can be end-labelled with biotin-labelled nucleotides.
12. The exact *in situ* hybridization technique described in this protocol has been used successfully to locate genes in polytene chromosomes of *Simulium damnosum, Drosophila melanogaster,* and at least eight different *Anopheles* species. Adequately flat chromosomes are harder to obtain from *Simulium* because of the high silk content of their salivary glands. This can be overcome by using half of one salivary gland and by tapping and pressing harder. Removal of the outer sheath of the *Simulium* salivary glands helps too. In *Drosophila* one pair of salivary glands is usually sufficient for a chromosome spread.

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CHAPTER THIRTY

Microdissection and microamplification of dipteran polytene chromosomes

Robert D. C. Saunders

30.1 INTRODUCTION

30.1.1 Polytene chomosomes

Polytene chromosomes were first described, in *Chironomus*, by Balbiani (1881). However it was not until the 1930s that their importance in genetic research was realized. Calvin Bridges (1935) created a map of *Drosophila melanogaster* third instar larval salivary gland polytene chromosomes that is not only still used to this day, but is the model for polytene maps of many other species. Polytene chromosomes are widespread within the class Diptera, and occur in various tissues in different species.

Polytene chromosomes represent a substantial amplification of chromosomal DNA, to a ploidy of at least 1000-fold in the case of *D. melanogaster* and perhaps Anopheline ovarian chromosomes.

The procedure described in this chapter is essentially a modification of the original method of microcloning (Scalenghe *et al.*, 1981; Pirrotta *et al.*, 1983), using the polymerase chain reaction instead of molecular cloning in phage lambda to recover DNA microdissected from polytene chromosomes (Saunders *et al.*, 1989).

30.1.2 Overview of the method

Figure 30.1 shows a flow chart presenting the stages of the procedure, illustrating the major steps. The methods described here include the preparation of chromosomes suitable for microdissection, the extraction of DNA from the microdissected fragments and its amplification using the polymerase chain reaction.

Most of the molecular biological steps are carried out in an oil chamber, to prevent evaporation. The oil chamber is a thick glass slide with a deep groove (Figure 30.2A). Narrow coverslips are laid across the groove, and the gap filled with buffer saturated paraffin oil, to prevent evaporation of reagents during the protocol. The molecular manipulations are carried out in minute drops hanging from the underside of cover-

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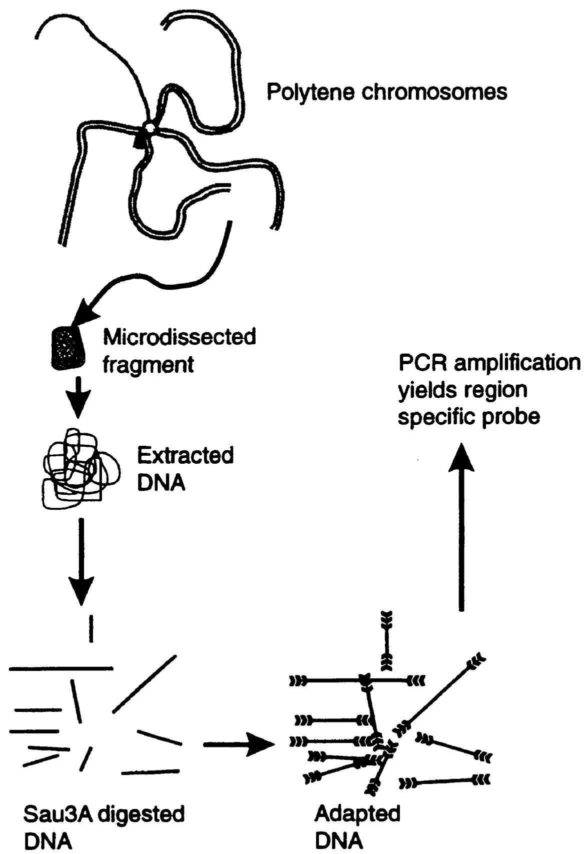


Figure 30.1 Microamplification flow chart. The figure schematically illustrates the major steps in the microamplification protocol. All except the PCR amplification step are carried out under the compound microscope.

slips. Generally, three coverslips are placed on the oil chamber at any one time, and microinstruments enter the chamber horizontally from the rear (Figs. 30.2A and B).

The choice of equipment will depend on that already available, since suitable microscopes, micromanipulators and microforges are expensive and unlikely to be obtained specifically for this purpose. The equipment used by the author consists of a fixed stage microscope and a free standing de Fonbrune micromanipulator. Microinstruments attached to the micromanipulator enter the oil chamber horizontally. It is of course possible to modify the setup to use a conventional stage focusing microscope with a stage mounted micromanipulator: such a system would require a different design of oil chamber.

Microdissected chromosome fragments contain a large proportion of protein, which is extracted by a combination of proteinase K digestion and phenol extraction, leaving relatively purified DNA in a microdrop of buffer. To reduce this high molecular weight DNA to manageable sized fragments suitable for PCR amplification, it must be digested with a restriction enzyme. Suitable restriction enzymes are those with four base pair recognition sequences: these generally reduce the DNA to an average size of about 300 bp. Additionally, it is convenient if the enzyme may be heat inactivated, eliminating the need for a further phenol extraction to eliminate its activity.

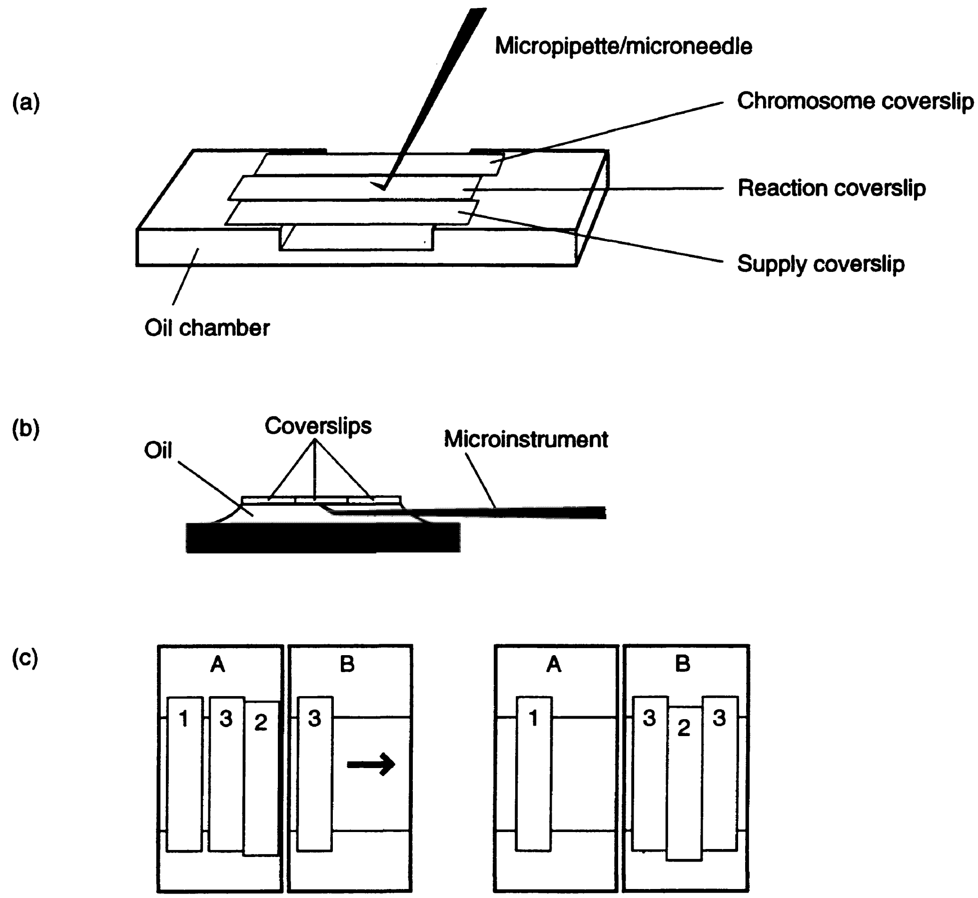


Figure 30.2 Oil chamber. (A) and (B) The arrangement of the oil chamber. The microinstrument enters the chamber below the coverslip, from the rear. Three coverslips are on the chamber: chromosome, reaction and supply coverslips. The groove spanned by the coverslips is filled with buffer saturated paraffin oil. (C) Switching coverslips between oil chambers. The supply coverslip is removed, and the reaction cover-slip (2) moved away from the chromosome coverslip (1), to the edge. Fresh unsiliconized coverslips (3) are placed as indicated. Oil is added to chamber B. The chambers are brought together and the two coverslips are slid over from chamber A. Fresh oil is added as required.

30.2 MATERIALS

30.2.1 Chromosome preparation

1. 0.7% NaCl.
2. 45% Acetic acid.
3. Glass slides.
4. Coverslips 18 × 18 mm (siliconized), 22 × 40 mm (unsiliconized).
5. Ethanol (70% and 100%).
6. Forceps: two pairs Dumont No 5 for dissection, one pair long for dipping slides in liquid nitrogen.
7. Liquid nitrogen.

30.2.2 Microscope and micromanipulator

1. Fixed stage compound microscope, with 10× and 40× dry phase objectives.
2. De Fonbrune micromanipulator. Alternative equipment may be used, though this may require deviations from the oil chamber arrangement described here. Micropipettes are used with a 10 mL syringe connected with rubber tubing *via* a three way valve. This arrangement allows the air in the syringe to be expelled without losing the contents of the micropipette.
3. Microforge.
4. Stereo dissection microscope.

30.2.3 DNA extraction and amplification

1. 200 μL glass capillaries. These capillaries are used to make the micropipettes and microneedles (Section 30.2.3a below).
2. Extraction buffer: 0.5 mg/mL proteinase K, 10 mM Tris-HCl pH 7.5, 10 mM NaCl, 0.1% SDS.
3. 4× R buffer: 40 mM Tris-HCl, pH 7.5; 400 mM NaCl; 40 mM $MgCl_2$; 40 mM β-mercaptoethanol.
4. Paraffin oil, IR spectroscopy grade, or similar. Paraffin oil equilibrated with 1× R buffer is required to fill the oil chamber.

(a) Glassware

1. 5 × 40 mm coverslips (cut from 20 × 40 mm coverslips). Both siliconized and unsiliconized coverslips are required.
2. Oil chamber. This is a 5 mm thick glass slide with a 3 mm deep groove cut in it (Figure 30.2). At least two of these are required.
3. Microinstruments: These are constructed from 200 μL capillaries using a microforge. In both cases they enter the oil chamber horizontally and require an angled tip to reach the underside of the coverslip. Micropipettes must have a 90° bend to allow the tubing from the syringe to be attached. Figure 30.3 illustrates the steps involved.
   Microneedles: Initially, the capillary is heated with a bunsen flame halfway along its length, and pulled to about one third original thickness. A second pull reduces the diameter to about 0.5 to 1 mm. Break the capillary and mount one half on the microforge, with the tip close to the element.
   Touch the tip to the heated element and pull it away while reducing the element current, yielding a fine tip extending about 2 mm at right angles to the microneedle body. Turn the needle through 180°C so that the bend is towards the element, and turn on the element current. Do not allow the needle to touch the element. As the glass melts, the needle tip will fall to 45°C from horizontal. Switch off the current and remove the needle. Prior to use, the needle's tip must be broken against the chromosome coverslip to give a sharp edge.
   Micropipettes: These are initially pulled as for the microneedles, except the narrowing of the glass should be close to one end. Break off the short end of the micropipette, and mount the capillary at 45°C to the microforge element as illustrated. Attach a small lump of adhesive putty as a weight. Do not allow the glass to touch the element. Heat the element, and the lower portion of the capillary

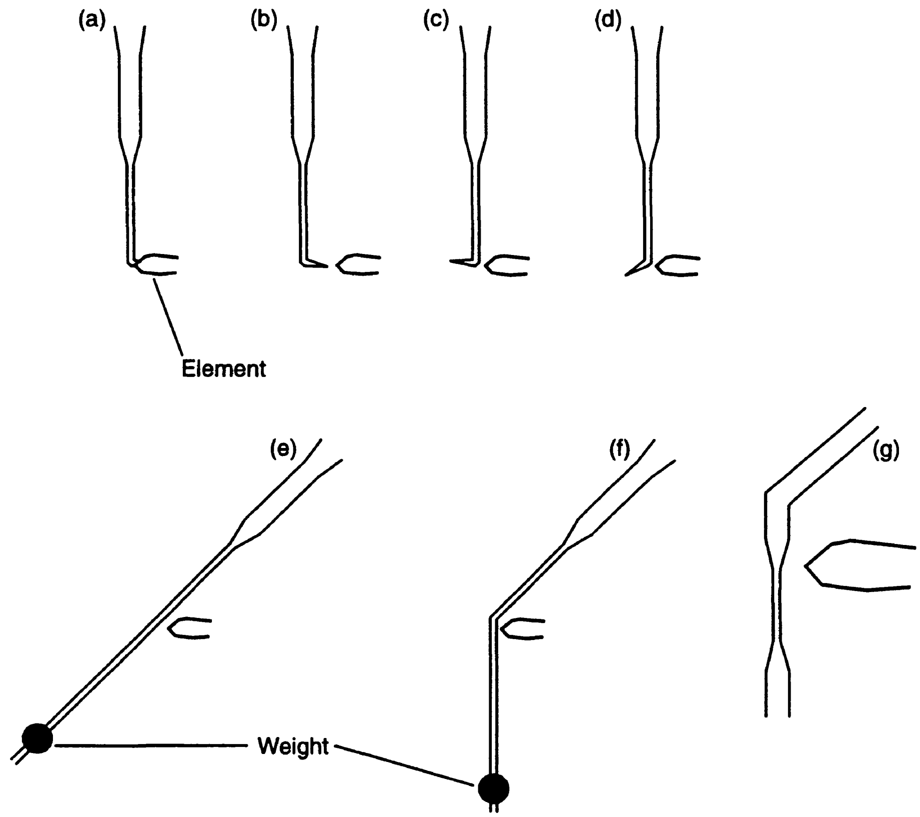


Figure 30.3 Microneedles and micropipettes. (A) The pulled capillary is brought into contact with the element, and melted. (B) By reducing the element current and moving the capillary away from the element, the melted glass is drawn to a point. (C) and (D) Turn the capillary round, then gently heat the element. As the glass melts, the tip of the capillary will drop. A bend of 45° to the horizontal is ideal. (E) The capillary is positioned over the element. (F) When the element is heated, the capillary will bend, and the lower portion will drop to the vertical. Care must be taken that the capillary does not touch the element. (G) Move the capillary up by about 3 mm. Heat the element. As the glass melts, the capillary will stretch and pull to give a narrow diameter neck. Turn off the element, remove the capillary and snap at the constriction.

will drop to vertical. Turn off the element. Raise the capillary by about 3 mm, and gently heat the element. The capillary will begin to stretch as the glass melts. Once the desired fineness has been attained, turn off the element, and break off the lower portion of the capillary. The end should be open. Using a bunsen flame, heat the wide end of the capillary and bend it into an L shape to provide the attachment point for the tubing.

Finally, siliconize the micropipette by filling it to 1 cm with siliconizing fluid (e.g. Repelcote). Leave for a few minutes, then rinse several times with sterile distilled water. Allow to dry.

(b) Enzymes

1. Proteinase K (Sigma).
2. *Sau*3A 50 U/μL (Amersham).
3. T4 DNA ligase, 1 U/μL (Boehringer).

(c) Oligonucleotides

Double-stranded adapter oligonucleotide. 10 μmol in 10 mM Tris-HCl, pH 7.5; 10 mM $MgCl_2$; 5 mM β-mercaptoethanol; 2 mM ATP (Section 30.3.2 gives details of preparation).

Oligonucleotide primer. 100 μM solution in sterile distilled water. Primers are discussed in section 3.2.

30.3 METHOD

30.3.1 Chromosome preparation

Good quality polytene chromosomes are found in various tissues in different Diptera. In the case of Anopheline mosquitoes the best are found in the ovaries of half-gravid females (Chapter 27). Whatever the tissue used, it is important that it is not exposed to acidic fixatives for more than two minutes. Longer fixation causes excessive damage to the DNA, compromising later molecular biological steps. The dissection and fixation steps are conveniently carried out on a plastic Petri dish lid, or a siliconized slide. All coverslips must be scrupulously clean and dust free. Clean them with a tissue moistened with ethanol, then blow the lint off with a photographer's air can (Kenair).

1. Prepare several clean coverslips (18 × 18 mm siliconized, and 22 × 40 mm) and glass slides.
2. Dissect the tissue from the insect in 0.7% NaCl. Transfer the tissue to a fresh drop of saline.
3. Place a drop of 45% acetic acid on a clean siliconized 18 mm coverslip on a slide. Transfer the tissue to the acetic acid. Incubate for about 90 s.
4. Touch the 22 × 40 mm coverslip to the drop to pick up the smaller coverslip, invert it and replace it on the slide. Now the large coverslip lies between the small coverslip and the slide.
5. Spread the chromosomes by tapping with a circular motion with a pencil point. Check that the chromosomes are spread sufficiently by examination with a dry phase microscope. A small amount of fix between the coverslip and the slide will hold the coverslips in place during steps 6 and 7.
6. Fold the slide in bibulous paper, press gently to remove excess fix, then squash hard. Immediately remove the slide from the paper and plunge it into liquid nitrogen. When the nitrogen stops boiling, retrieve the slide, quickly remove the small coverslip with a scalpel blade, prise the large coverslip off the slide and immediately plunge it into 70% ethanol.
7. After 5 min, pass the coverslip through a further 5 min in fresh 70% ethanol, 5 min in 100 % ethanol, then air-dry.
8. Examine the chromosomes by dry phase contrast. Good chromosomes will be flat and grey, while poor chromosomes are refractile and shiny. Before use, the coverslip must be trimmed with a diamond pencil to a 5 mm wide strip.

30.3.2 Oligonucleotide preparation

Two oligonucleotides are required for this protocol. One is a 24-mer, the other a 20-mer. Typical sequences are as follows:

5′ GATCAGAAGCTTGAATTCGAGCAG 3′
5′ CTGCTCGAATTCAAGCTTCT 3′

These oligonucleotides are complementary, and when annealed have a *Sau*3A cohesive end. In addition, the 20-mer serves as the PCR amplification primer.

1. Make a solution of the two oligonucleotides, each at a concentration of 20 μM in 20 mM Tris-HCl, 20 mM $MgCl_2$. A volume of 50 μL is typical. Incubate at 80°C for 5 min, then allow to cool slowly to RT.
2. Add the following:
   46 μL sterile distilled water
   2 μL 100 mM ATP
   2 μL 250 mM β-Mmercaptoethanol
   The adapter oligonucleotides are ready for use.

30.3.3 Chromosome microdissection

1. The **chromosome coverslip** is positioned over the groove of the oil chamber, ensuring the chromosomes are on the lower surface, and the space filled with paraffin oil. A siliconized 5x20 mm coverslip is placed adjacent to it. This is the coverslip from which the reaction drops will hang (**reaction coverslip).** Add further oil if necessary. The chromosome coverslip may be scanned for good chromosomes and their position recorded at this stage. Generally this is best done some time prior to the rest of the procedure, as this is the most time-consuming part of the protocol. Only after chromosomes of suitable quality have been located should one proceed to step 2.
2. Place about six drops of extraction buffer (about 0.2 μL each) on a siliconized 5 × 40 mm coverslip and place it on the oil chamber next to the reaction coverslip. This coverslip is the **supply coverslip.** Add further oil if necessary.
3. Place the oil chamber on the microscope stage, with the chromosome coverslip towards the micromanipulator. Mount a micropipette on the micromanipulator and arrange it so that its tip is upwards and focused under the 10× objective. Using the stage controls, move the oil chamber under the objective, ensuring that the micropipette will collide with neither the oil chamber, nor the coverslips. Focus on the micropipette tip, then move the oil chamber until the supply drops of extraction buffer are in view. Partly fill the pipette with oil, then pull up as much extraction buffer as possible. The oil prevents an air/buffer interface. Move the chamber so that the pipette tip is under the reaction coverslip, and dispense a row of at least 6 drops about 1 mm apart, noting their stage coordinates. Using an eyepiece graticule, the volume of the drop can be estimated. Each reaction drop should be approximately 1 nL.
4. Remove the micropipette and replace it with a microneedle. Locate a region devoid of chromosomes, and then break the tip to generate a sharp edge. Now bring the needle tip to the chromosome region of interest. By raising the microneedle, the tip first comes into contact with the glass, then as the needle deforms will push along the coverslip. It is this action which should be used to dissect chromosome regions rather than the stage or micromanipulator controls. The chromosome fragment will be pushed to one side of the chromosome, and can be picked up by touching it with the needle tip.

5. Making sure the needle tip is below the coverslips, move the stage till the needle tip approaches a reaction drop. By gently moving the stage so that the tip of the needle enters the drop, the fragment will either be seen to fall into the drop, or will disperse more slowly. In the latter case, it will become quite viscous.
6. The chromosome fragment will disperse in the reaction drop very quickly, but the oil chamber should be incubated in a humid box at RT for about 30 min before proceeding.

30.3.4 DNA extraction

The extraction buffer contains proteinase K and SDS, to disrupt the microdissected chromosome fragment. The reaction drops are subsequently extracted with phenol equilibrated with 1× R buffer, serving two purposes: firstly to inactivate the proteinase K, and to alter the buffer composition towards that of buffer R, suitable for restriction enzyme digestion.

1. Replace the extraction buffer supply coverslip with a supply coverslip carrying buffer saturated phenol. This coverslip should not be siliconized. Make sure there is an aqueous drop within the phenol. Note that the oil/phenol interface can be hard to see.
2. Using a fresh micropipette, draw up a volume of phenol. Dispense approximately 4 vol phenol onto each reaction drop. It will form an outer layer. Incubate at RT for 2–3 min then remove as much as possible, without disturbing the aqueous phase.
3. Repeat step 2 twice more. Remove as much phenol as possible from the reaction drops.
4. Fill a micropipette with chloroform, then mount it on the micromanipulator (supply drops of chloroform are impossible, since chloroform is miscible with paraffin oil). Bring the pipette tip close to the first reaction drop, and blow a stream of chloroform onto the drop, removing all traces of phenol. Take care not to dislodge the reaction drop. Continue until all reaction drops have been chloroform extracted.
5. The reaction coverslip must now be transferred to a fresh oil chamber, since the original is now contaminated with phenol and chloroform (Figure 30.2). Set up a second oil chamber with a single unsiliconized coverslip. Remove the phenol supply coverslip, and bring the second oil chamber adjacent to the first. The reaction coverslip can now be slid across to the new oil chamber, followed by a fresh, unsiliconized coverslip.

30.3.5 *Sau*3A digestion

1. Remove the first unsiliconized coverslip, and replace with a siliconized supply coverslip bearing several drops of *Sau*3A. The enzyme should be high concentration and diluted 1:1 in 4× R buffer, with a final concentration of 20–25 U/μL.
2. Fill a fresh micropipette with *Sau*3A, and dispense a drop of equal volume adjacent to the first reaction drop. Allow the two drops to fuse (this may require gentle persuasion). Repeat with the remaining reaction drops.

3. Place the oil chamber in a humid box and incubate for 90 min at 37°C.
4. Inactivate the *Sau*3A by incubating the oil chamber, in the humid box, at 70°C for 20 min. The oil becomes less viscous at high temperatures, so care must be taken to ensure it does not leak from the oil chamber.
5. Allow the oil chamber to cool within the humid box to RT.

30.3.6 Adapter ligation

1. Prepare the adapter-ligase mix, by mixing 1 μL adapter solution with 1 μL ligase and place on ice.
2. Prepare a siliconized supply coverslip carrying several supply drops of ligase-adapter mix and place it on the oil chamber.
3. Using a fresh micropipette, dispense an equal volume of adapter-ligase mix adjacent to the first reaction drop and allow the drops to fuse. Repeat with the remaining reaction drops.
4. Place the oil chamber in a humid box, and incubate at 4°C overnight.

30.3.7 PCR amplification

1. Allow the oil chamber to come to RT.
2. Prepare a series of sterile tubes appropriate to the thermal cycler to be used, by labelling them and adding 10 μL sterile distilled water. Place these tubes on ice.
3. Place the oil chamber in a plastic Petri dish. Fill the dish with paraffin oil until the level reaches the top of the oil chamber, taking care not to sweep the reaction drops off their coverslip.
4. Slide the unsiliconized coverslip off the oil chamber, and remove it. Slide the reaction coverslip to the edge of the oil chamber then carefully push it off the chamber, invert it and place it on the bottom of the Petri dish, under the oil with the reaction drops uppermost. This is a manoeuvre that should be practised! Place the dish under a stereo microscope.
5. Using a P20 micropipette or similar device, take up 2 μL water from the tube corresponding to the first reaction drop. Pipette some of this water onto the first reaction drop, and draw it and the drop contents into the pipette tip. Withdraw the pipette and expel the reaction into the remaining water in the tube. Replace the tube on ice. Repeat for the remaining reaction drops.
6. Set up the PCR reactions by adding the following to each tube:

10× PCR Buffer	10 μL
10 mM dNTP solution*	2 μL
Primer, 100 μM	0.5 μL
Sterile distilled water	77 μL
Taq polymerase, 5 U/μL	0.5 μL
Total	90 μL

*10 mM in each nucleoside triphosphate.

Remember to include a control reaction with no added template DNA to check there is no contamination of reagents.

7. Pipette a layer of paraffin oil over the tube contents, and place the tubes in the thermal cycler. A suitable programme using the primer described is as follows:

37°C 5 min
55°C 5 min
72°C 5 min
94°C 1 min
55°C 1 min
72°C 3 min
Followed by 30 cycles of

8. After the programme is complete, analyse 5 μL of each sample by electrophoresis on a 1% agarose gel. A suitable size marker would span 100 bp to 1 kb. The amplified DNA should run as a smear of 200 bp to 900 bp in size. However, in some cases a degree of banding may be visible superimposed upon the smear. This is generally due to the presence of repetitive DNA containing *Sau*3A restriction sites. An example is the *D. melanogaster* X chromosome specific 359 bp satellite DNA, which has 2 *Sau*3A sites.

NOTES

Choice of restriction enzyme

A variety of restriction enzymes may be used for the fragmentation of microdissected DNA, but the selected enzyme must have the following characteristics:

1. A four nucleotide recognition site, yielding cohesive termini, preferably of four residues.
2. It should be heat inactivatable. If this is not the case, additional phenol and chloroform extractions must be performed prior to adapter ligation.
3. The enzyme must be compatible with the experimental organism's DNA methylation state. *Sau*3A is ideal in this regard, as it cleaves DNA irrespective of DNA methylation. Note that if an enzyme other than *Sau*3A is used, R buffer must be replaced with a buffer of suitable composition, and the sequence of the double-stranded adapter oligonucleotide must be changed to match the cohesive termini produced by the new enzyme.

Analysis of amplified material

Once DNA has been successfully amplified, it must be experimentally confirmed that it is derived from the region microdissected. This is best achieved by *in situ* hybridization (Chapter 28). This will also be informative as to the content of repetitive DNA within the amplified DNA. Care must be taken in this analysis; because the amplified probes are complex, signals due to transposable elements can be very weak.

Uses of microamplified DNA

Region-specific probe
DNA amplified from microdissected chromosome fragments is principally useful as a region specific probe with which clones may be selected from a genomic library.

The presence of repetitive DNA within the microdissected DNA will yield a probe that will identify clones originating from outside the microdissected interval. This may be overcome in several ways. First, if only a few clones are concerned, each may be checked by *in situ* hybridization to verify their primary site of origin. In general, the primary site of origin is discernible even when the clone contains a dispersed middle repetitive sequence such as a transposable element. Second, the genomic library may be prehybridized with unlabelled total genomic DNA to suppress signals due to repetitive DNA. A third approach, and one that has been successfully used in *Drosophila* genome mapping (Saunders *et al.*, 1989; Sidén-Kiamos *et al.*, 1990; Madueño *et al.*, 1995) has been to perform the microdissection on a related species with near identical polytene chromosome banding patterns. A low resolution genome map of *Anopheles gambiae* (Zheng *et al.*, 1991) consisted of amplified pools of microdissected DNA.

Source of microsatellite clones

It is possible to clone a pool of amplified fragments and screen the resulting mini-library for clones containing microsatellites. The advantages of this approach is that the investigator may target specific regions of the genome for this analysis. Some microsatellites used in mapping the *An. gambiae* X chromosome (Zheng *et al.*, 1993) were identified in this way.

Chromosome painting

Microdissected and amplified DNA may be used for chromosome painting, a technique using fluorescent *in situ* techniques to label particular chromosome segments. This would be particularly useful in identifying chromosomes and chromosome regions in metaphase chromosome preparations. It may be potentially useful in determining polytene chromosomal homologies between related mosquito species.

Problem solving

Obviously, in a complex procedure there are many steps where a problem may lead to the complete absence of a product. In practice, all the biochemical steps can be checked using known starting material, for example a single *Bam*HI fragment. Repeated failure can indicate that the chromosomes have been fixed for too long. This results in damaged DNA. Some protocols for preparation of polytene chromosomes for other applications require dissection of tissue in dilute organic acid. Such steps are to be avoided.

Always check the amplification product by *in situ* hybridization, as cross-contamination between reaction drops can occur. Ideally cross-contamination can be avoided by using a fresh micropipette for each buffer, and for each reaction drop. However, this would require up to 10 micropipettes for each stage in a typical microdissection session.

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Part Five
Insect Identification Techniques

CHAPTER THIRTY ONE

RAPD-PCR and SSCP analysis for insect population genetic studies

William C. Black IV and Nancy M. DuTeau

31.1 INTRODUCTION

Population genetic studies require analysis of many individuals with multiple genetic markers. Prior to the development of the polymerase chain reaction (PCR) insect population geneticists relied almost exclusively on biochemical polymorphisms (Tabachnick and Black, 1996) as genetic markers. Random Amplified Polymorphic DNA (RAPD) and Single-Strand Conformation Polymorphism (SSCP) analysis are two PCR-based techniques developed in the last 5 years that allow a virtually unlimited number of genetic markers to be amplified in individual insects. Both techniques are simple, rapid, safe and relatively inexpensive. They offer the insect geneticist an unprecedented ability to examine many genetic markers simultaneously. This improves resolution of spatial and seasonal genetic differentiation among populations and increases the accuracy and precision of gene flow rate estimates. Monitoring segregation of markers distributed throughout a genome also allows for intensive examination of linkage disequilibrium. RAPD and SSCP markers have already been used in population genetic studies (Puterka *et al.*, 1993; Tabachnick and Black, 1996; Apostol *et al.*, 1996; Norris *et al.*, 1996) and in genetic fingerprinting (Apostol *et al.*, 1993, 1994). They have also proven useful in detection and identification of cryptic species (Black *et al.*, 1992; Wilkerson *et al.*, 1993; Kambhampati *et al.*, 1992; Ballinger-Crabtree *et al.*, 1992; Hiss *et al.*, 1994; Black and Munstermann, 1996).

31.1.1 Properties of RAPD markers

RAPD-PCR uses a single short (10 bp) primer of arbitrary sequence with a minimum GC content of 60% to amplify many genomic regions simultaneously (Williams *et al.*, 1990). During RAPD-PCR, the primer is annealed to genomic template DNA at a low temperature (35°C). In theory, the primer anneals to many regions of the genome simultaneously. However, geometric amplification only occurs in those regions in which the 3′ end of the annealed primers face one another on opposite strands and are no more than 3 kb apart. These conditions suggest that the primer annealing sites must be inverted repeats. Moderate and highly repetitive DNA segments in cen-

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tromeric, telomeric and heterochromatic genomic regions are rich in inverted repeats, as are various classes of dispersed repetitive and mobile elements. RAPD-PCR is biased in its amplification of these repetitive regions but amplifies many unique regions as well (Williams *et al.*, 1990).

Polymorphisms at regions amplified by RAPD-PCR are typically manifest as the presence or absence of a band among individuals (Figure 31.1). Many studies have shown that most (>90%) amplified RAPD bands are inherited in a Mendelian fashion as dominant alleles. This means that an individual displaying a band may possess two copies (homozygous dominant) or a single copy (heterozygous dominant) of an amplifiable allele at a RAPD locus. A homozygous recessive individual displays no band. This is a negative feature of RAPD markers for population genetics because it prevents tests of random mating in populations. These tests require complete genotype information. However, many of the population genetic analyses of spatial differentiation (Wright's F_{ST} and genetic distances) can still be applied to RAPD markers and methods for testing for linkage disequilibrium among RAPD loci have been developed (Lynch and Milligan, 1994; Black, 1996; Apostol *et al.*, 1996). Computer programs for population genetic analysis of RAPD polymorphisms are available (Note 1). RAPD-PCR has also been criticized for lack of reproducibility. However, this problem can be overcome with careful standardization of techniques and reagents (Note 2).

Advantages of RAPD markers include: quick detection of usually 50–60 polymorphic DNA markers in 4–5 PCR reactions, nonradioactive detection of products, speed (a group of 50–60 insects can be analysed within 24–48 h), and the ability to carry out the procedure without any prior sequence information. Analysis of RAPD markers has traditionally been performed on 1.2–1.5% agarose gels (Figure 31.1a). In this chapter we demonstrate that band mobilities are easier to compare and more information can be extracted from RAPD products run on vertical PAGE gels and detected with silver staining (Figure 31.1b).

31.1.2 Properties of SSCP markers

Single-Strand Conformation Polymorphism (SSCP) analysis is based on the principle that electrophoretic mobility of a single-stranded DNA or RNA molecule in a nondenaturing gel is dependent upon both its size and shape. A number of stable shapes or conformations are formed when secondary base pairing occurs among nucleotides on a single DNA strand. The length, location and number of intra-strand base pairs determines secondary and tertiary structure of a conformation. Point mutations that affect intra-strand interactions may therefore change the shape of a molecule and thus alter its impedance during electrophoresis. In principle, the mobility of a renatured single-stranded DNA molecule should be sensitive to point mutations. Orita *et al.* (1989) developed SSCP analysis to test this principle and showed that intra-strand interactions are highly sensitive to the primary sequence of the molecule. Hayashi (1991), in an extensive study of the sensitivity of the technique, concluded that SSCP could detect 99% of point mutations in DNA molecules 100–300 bp in length and 89% of mutations in molecules of 300–450 bp in length.

SSCP analysis is methodologically simple. Double-stranded DNA molecules are denatured to single-strands with heat and then plunged into ice to promote the formation of intra-strand complexes over renaturation of the complementary strands.

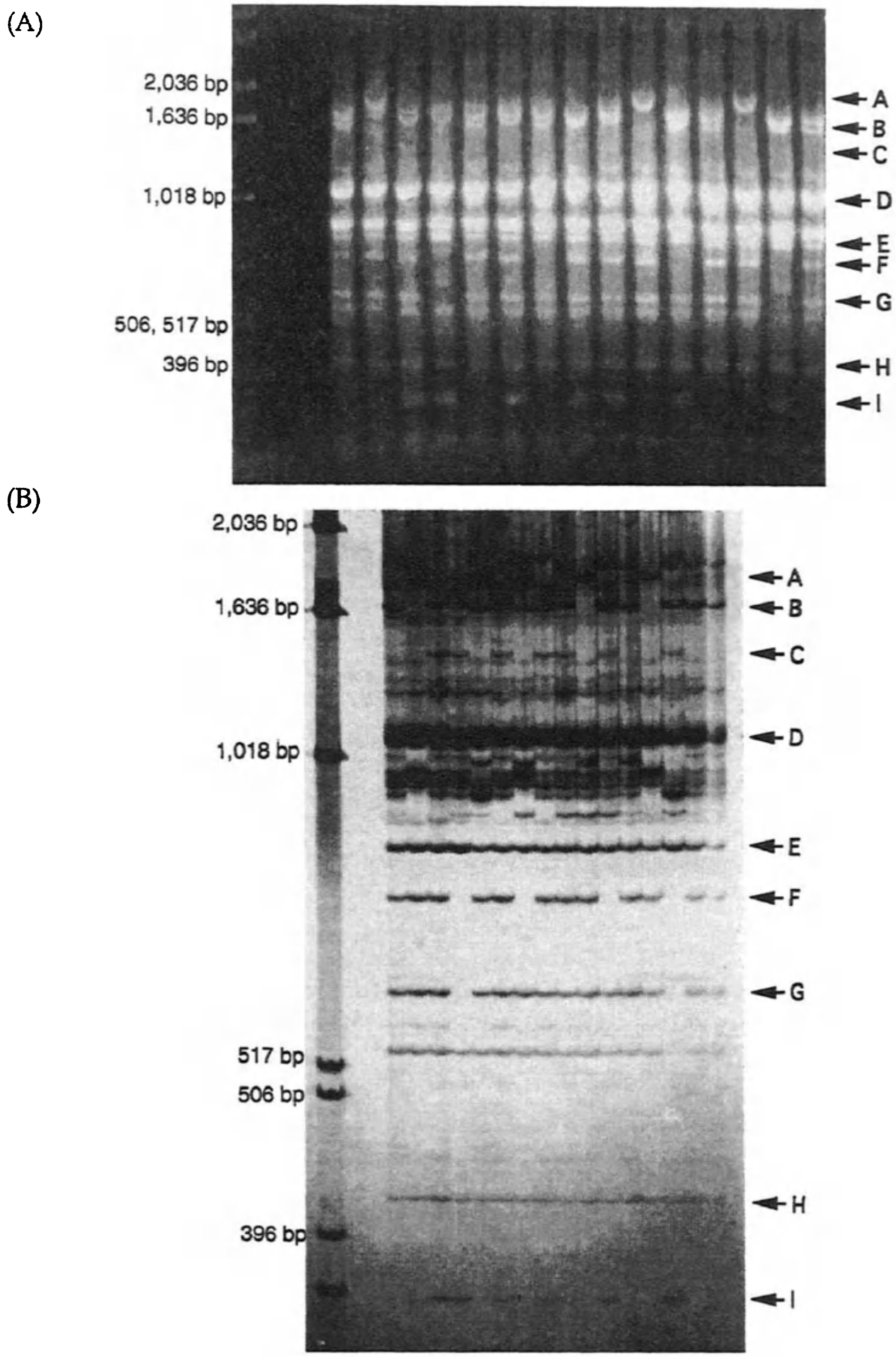


Figure 31.1 RAPD-PCR analysis of an *Aedes triseriatus* family using the C-13 primer (5′-AAGCCTCGTC-3′) (Operon Technologies Inc., Alameda, California). (A) Analysis of RAPD-PCR products on a 1.5% agarose gel. (B) Analysis of RAPD-PCR products on a 38 × 50 cm polyacrylamide gel. DNA was visualized with silver staining. The last lane in Figure 31.1B is a duplicate loading of the previous lane. In both gels, the BRL 1 kb ladder (Gibco-BRL Life Sciences Inc., Gaithersburg, Maryland) were used as size markers and polymorphic bands are indicated and matched across gels with letters A-I. Note that band 'C' is barely visible on the agarose gel but is easily visualized on the acrylamide gel. Note also that the series of bands resolved between bands 'D' and 'E' on the acrylamide gel are resolved as only a single band on agarose. In fact, these represent codominant alleles at a single locus.

Two classes of single-stranded DNA (ssDNA) are formed. These are denatured single-strands (DSS) and renatured single-strands (RSS). The mobility of DSS is dependent on the molecular weight of the molecule and is thus sensitive to insertions and deletions. Mobility of RSS is dependent upon the conformation formed by intra-strand base pairing. This conformation and the mobility of RSS is dependent upon the primary sequence of the molecule. Often a DNA sequence can have several different stable conformations.

Renatured products are loaded onto a native polyacrylamide gel and electrophoresis is performed at, or below, RT to ensure that intra-strand base pairs are not disrupted. The gel is then silver stained to detect the mobility of the different DNA conformations. A typical SSCP gel is shown in Figure 31.2.

SSCP is a sensitive tool for population genetic analysis of discrete genetic markers. It has the potential to detect single point mutations and is therefore more sensitive than RFLP analysis. Nuclear or mitochondrial markers can be amplified in individual insects and subjected to SSCP analysis. SSCP gels can then be scored to determine the amount of genetic variability of a marker in a population. Variants can be sequenced to determine the precise mutations associated with SSCP polymorphisms. SSCP analysis can be used to screen large numbers of individuals and sequence data need only be collected from the variants. This strategy was applied recently in a population genetics study of the 16S and 12S mitochondrial rDNA genes in the Lyme disease vector *Ixodes scapularis* (Figure 31.2) (Norris *et al.*, 1996). In this study SSCP analysis was found to be 100% sensitive to all point mutations in a 300 bp region of the 16S gene.

In analysis of nuclear markers SSCP can be used to detect variation at both alleles at a locus. Polymorphisms identified by SSCP therefore segregate as co-dominant markers. This means that, unlike RAPD-PCR, SSCP can be used to test for random mating in populations. The only major disadvantage associated with the SSCP analysis is the requirement for sequence information with which to construct specific primers to amplify unique mitochondrial and nuclear genes. However, primers are now available for a large number of mitochondrial and nuclear genes in arthropods (Simon *et al.*, 1994; Brower and DeSalle, 1994). Just as important, many genes have been cloned and identified in vector species. It will be exciting to determine the extent of genetic variation in these sequences. SSCP analysis promises to become a powerful tool in this endeavour.

31.2 MATERIALS

31.2.1 DNA isolation from individual arthropods

1. Method 1: Grinding buffer (0.1 M NaCl, 0.2 M sucrose, 0.1 M Tris-HCl pH 9.1, 0.05 M EDTA, 0.05% SDS). Make up grinding buffer and store frozen (–20°C) in 1 mL aliquots.
2. 8 M Potassium acetate.
3. Method 2 (CTAB): Grinding buffer (100 mM Tris-HCl pH 8.0, 1.4 M NaCl, 0.02 M EDTA, 2% CTAB (hexadecyltrimethylammonium bromide), 0.2% β-mercaptoethanol). Store at 4°C.
4. TE: 0.01 M Tris-HCl pH 8.0, 1 mM EDTA.
5. 70%, 95%, 100% Ethanol stored at –20°C.

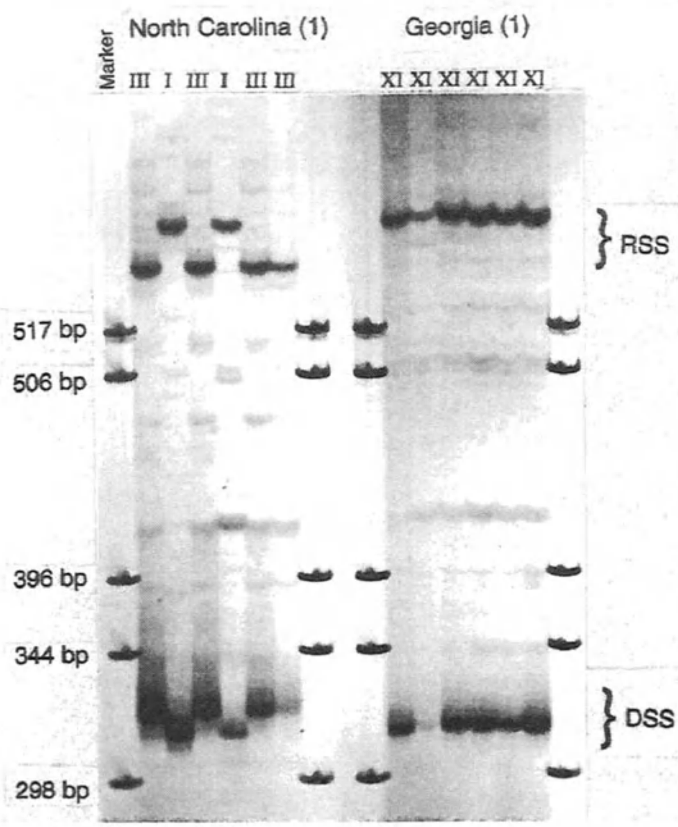


Figure 31.2 Single-strand conformation polymorphism (SSCP) analysis of 300 bp of the 16S mitochondrial rDNA gene in the tick *Ixodes scapularis*, the primary eastern North American Lyme disease vector. The BRL 1 kb ladder (Gibco-BRL Life Sciences Inc., Gaithersburg, Maryland) was used for size markers. The 16S gene region was amplified from six ticks removed from a dog in Currituck Co., North Carolina. Two distinct haplotypes, scored as I and III are present. The next set of lanes are 16S PCR products from six ticks from Bulloch Co., Georgia and are from a colony maintained at Georgia Southern University. They are haplotype XI. Norris *et al.* (1996) provides details on haplotype designations, geographical distributions, sequences, and phylogenetic relationships. (RSS, renatured single-strands and DSS, denatured single-strands).

31.2.2 PCR

1. Double glass distilled deionized water or 18.3 MΩ deionized ('Ultrapure') water, autoclaved and filtered with 0.22 μm filters. Stored frozen at –20°C in 1 mL aliquots.
2. 10× buffer: 500 mM KCl, 100 mM Tris-HCl pH 9.0, 15 mM $MgCl_2$, 0,1% gelatin (w/v) and 1% Triton X-100.
3. dNTP mix: 20 mM of dATP, dCTP, dGTP, dTTP. Store in 10–30 μL aliquots in the –70°C freezer and thaw immediately prior to use.
4. RAPD-PCR primers: diluted to a concentration of 500 pmol/μL (Note 3 on primer selection).
5. *Taq* polymerase.

31.2.3 PAGE gels

1. Bind silane: γ-methacryloxy-propyl-trimethoxysilane, Sigma Chemical Co.

2. Sigmacote: Sigma Chemical Co.
3. Plate cleaning solution: 10 M NaOH, 0.2 M SDS. The SDS remains as an emulsion.
4. 30% acrylamide gel solution (100 mL): 29.4 g acrylamide, 0.6 g N,N'-methylene bisacrylamide dissolved in 100 mL ddH_2O.
5. 5× TBE (1 L): 54 g Tris base, 27.5 g Boric acid, 20 mL 0.5 M EDTA, pH 8.0.
6. TEMED: N,N,N',N'-Tetramethylethylenediamine, Sigma Chemical Co.
7. Ammonium persulfate: 25% (w/v) solution.
8. RAPD gel loading buffer: 1× TBE containing 20% sucrose, 0.05% bromophenol blue and 0.05% xylene cyanol.
9. SSCP gel loading buffer: 10 mM NaOH, 95% formamide, 0.05% bromophenol blue and 0.05% xylene cyanol.

31.2.4 Silver staining

1. 10–12 L ddH_2O or 18.3 MΩ deionized ('Ultrapure') water. This is used to make all solutions described below and in all washes. (Note 4).
2. When working with large (38 × 50 cm gels), 2 L of each solution listed below are required. For smaller gels (e.g. 20 × 20 cm), only 500 mL of each solution is needed.
3. Gel fixative: 10% acetic acid.
4. Staining solution: 0.15% (w/v) silver nitrate and 0.15% (v/v) 37% formaldehyde.
5. Developer: 3% sodium carbonate chilled to approximately 4°C. Immediately prior to placing the gel in the developer, 37% formaldehyde and sodium thiosulfate are added to concentrations of 0.15% of 37% formaldehyde and 0.0002% respectively. Work from a 1% sodium thiosulfate stock.

31.3 METHODS

31.3.1 DNA isolation from individual arthropods

There are a number of methods available for isolating DNA from individual arthropods (Chapters 14, 16 and 32). We have found the two methods described below to be effective for RAPD-PCR and SSCP analysis.

(a) Method I

The first method originates from Dr Pat Romans' laboratory at the University of Toronto and is a modification of the Coen *et al.* (1982) procedure. It is used with many types of arthropods.

1. Place individual insects in 1.5 mL tubes with 25 μL grinding buffer. Grind with at least 10 turns of a pestle (we use the Kontes blue plastic pestles). Continue grinding until you can't see any insect parts. Wash the pestle with an additional 25 μL grinding buffer. Place the contaminated pestle in 1 M HCl. Before these are used again they should be rinsed several times, washed in soapy water, rinsed in distilled water and autoclaved. (These precautions to prevent contamination are only really necessary for PCR-based analysis.)

2. Briefly microfuge to get all homogenate down into the bottom of the tube. This is important!
3. Incubate homogenate at 65°C for 30 min.
4. While tubes are still warm, add 7 μL 8 M KAc. Mix by tapping.
5. Incubate on ice for a minimum of 30 min to precipitate SDS.
6. Centrifuge at 14 000–17 000 g for 15 min in a microcentrifuge. Transfer supernatant to fresh 1.5 mL tubes. Try not to carry over any SDS precipitate.
7. Add 100 μL 100% ethanol (EtOH) and incubate at RT for 5 min to precipitate nucleic acids (you may also stop at this point, putting the tubes at –20°C overnight). Microfuge at 14 000–17 000 g for 15 min.
8. Carefully remove EtOH so that the DNA pellet remains in tube. The pellet will often appear as a smear and as often you may not see anything at all. Do not be discouraged! Add 100 μL 70% EtOH, tap tube and centrifuge at maximum speed for 5 min. Carefully remove the EtOH. Add 100 μL 100% EtOH, tap and centrifuge at maximum speed for 5 min. Carefully remove the EtOH. Air-dry the pellets or use a Speed Vac.
9. Resuspend the pellet in at least 200 μL TE. This DNA will be stable in the refrigerator or freezer for up to 6 months and often longer.

(b) Method II

The second method is commonly called the CTAB method (Rogers and Bendich, 1985). It has proven useful for arthropods with tough cuticles, such as ticks or in insects in which the gut may contain inhibitors of PCR reactions.

1. Place specimen in a sterile 1.5 mL microcentrifuge tube, add 500 μL of CTAB buffer and homogenize with Kontes pestles as described above (Section 31.3.1a). Alternatively, we seal ticks in small (3 cm^2) bags with 100 μL of CTAB buffer, freeze the bag for 15 min at –70°C, remove it to a table top and immediately smash the bag contents with a large, heavy hammer. Slice open the bag, pipette the contents into a 1.5 mL tube and wash the remainder of the carcass from the bag with 400 μL CTAB buffer.
2. Vortex to mix the fragments with the buffer and incubate at 65°C for 45–60 min. Vortex once or twice midway during the incubation.
3. Centrifuge for 2 min in a microfuge at maximum speed. Remove the supernatant liquid to a new microcentrifuge tube on ice. Try not to transfer the debris. Add 200 μL CTAB to the pelleted debris and vortex. Incubate at 65°C for 20–30 min.
4. Centrifuge for 2 min, maximum speed. Remove the supernatant to the supernatant from step 3.
5. Add 700 μL of chloroform:isoamyl alcohol (24:1) to the combined liquid and vortex to mix. *Hold the lid down tightly during this step!* Centrifuge for 10–15 min to obtain a clean separation of the layers.
6. Pipette the aqueous (upper) layer into a clean microcentrifuge tube. Avoid transferring any part of the interface. Add 400 μL of cold isopropanol (–20°C) and mix gently. Place at 4°C (NOT –20°C) for at least 30 min.
7. Centrifuge for 20–30 min in a microcentrifuge at 4°C at maximum speed.
8. Carefully remove the supernatant and wash the pellet in 700 μL cold 70% ethanol. Tap the tube until the pellet comes free from the bottom of the tube. Centrifuge for 5 min, maximum speed. Carefully remove the supernatant and

wash the pellet in cold 100% ethanol. Centrifuge for 5 min, maximum speed. Air-dry the pellet or place it in a speed-vac. Add at least 200 μL sterile TE buffer to pellet and let it resuspend overnight at 4°C. DNA can be stored at 4°C for short-term use or at –20°C for long-term storage.

31.3.2 Setting-up and running RAPD-PCR reactions

Aerosol-resistant or 'blocked' pipette tips are used in all steps. Gloves are worn while setting up PCR reactions and while handling all reagents. Make sufficient 1× buffer for the number of reactions to be performed. Making a single large 1× buffer greatly reduces variation among PCR reactions due to pipetting and handling error. We get the best results in 50 μL reactions but 25 or 100 μL reaction volumes are also commonly used.

1. For each reaction desired, pipette 43.5 μL filtered, sterilized ddH_2O, 5 μL 10× buffer, 0.5 μL 20 mM dNTPs and 50 pmol of primer (or each primer for targeted PCR) into a single sterile tube (Note 2). A 1.5 mL tube generally holds enough 1× buffer for 30 reactions (50 μL/reaction). For large populations, 50–60 individuals might be analysed and sterile 15 mL conical tubes are used instead.
2. Aliquot the 1× buffer in 48 μL aliquots into 500 μL or 250 μL sterile tubes. Add two drops (approximately 25 μL) sterile light mineral oil into each tube with a plugged and sterilized Pasteur pipette.
3. Close the tops of the tubes and either place them on a transilluminator or into a ultraviolet chamber emitting short wave ultraviolet light (260–320nm) for 10–15 min. This step is very helpful in destroying contaminating template DNA. Change gloves at this point.
4. Template DNA (20–50 ng in 1.0–2.0 μL TE) is added through the oil to the 1× mixture (Note 5 concerning template DNA amounts).
5. Tubes are placed on a thermal cycler and heated to 95°C for 5 min. Change gloves again. The temperature is then reduced and held at 80°C while 0.5–1 U of *Taq* polymerase are pipetted into each tube (See Note 6).
6. Begin thermal cycling. Conditions commonly used are:

Step 1: (denaturation)	92°C for 1 min
Step 2: (annealing)	35°C for 1 min
Step 3: (gradual extension)	Increase temperature 1°C/8 s to 72°C.
Step 4: (complete extension)	72°C for 2 min.
Cycle to step 1, 45 times.	
Step 5: (final extension)	72°C for 7 min.
Step 6: (overnight storage)	Hold at 4°C.

   This programme usually requires 16 h for completion and is most conveniently set up and run late in the day and the products removed and analysed on the subsequent day.
7. Pour a 1.5% 1× TBE agarose gel to determine if RAPD products were obtained. Add ethidium bromide (EtBr) to the gel (5 μL of 10 mg/mL EtBr per 100 mL gel solution).
8. Mix 5 μL of each reaction with 2.5 μl of RAPD loading buffer. Load size markers in the range of 200–3000 bp. Observe gel over an ultraviolet light to determine if RAPD products have been successfully amplified. Proceed to 3.4 if products are obtained.

31.3.3 Setting up and running targeted PCR reactions for SSCP analysis

PCR that attempts to amplify a specific region of the nuclear or mitochondrial genome is referred to as 'targeted' PCR. Simon *et al.* (1994) provides a catalogue of primers used in amplifying regions of the mitochondrial genome. Brower and DeSalle (1994) list potential regions of the nuclear genome. Most of the steps in 31.3.2 are followed in setting up targeted PCR reactions (for differences see Note 7).

31.3.4 Pouring, loading and running vertical PAGE gels for RAPD or SSCP analysis

1. Treat the outer (usually unnotched) glass plate with bind silane to adhere the gel to the plate for staining.
2. The adhesive glass plate is thoroughly scrubbed with an abrasive plastic pad and plate cleaning solution. It is then rinsed with tap water followed by a distilled water rinse and dried with lint free tissues (LFTs) (15 × 17 in Kim wipes). The plate is then sprayed with 95% ethanol (EtOH) and wiped clean with LFTs.
3. Bind silane (5 μL) is mixed in 1 mL of 95% EtOH and 0.5% glacial acetic acid (v/v). *You must wear gloves when handling bind silane.* The bind silane solution is poured onto a small cotton gauze pad and wiped evenly across the surface of the adhesive glass plate. Use a consistent vertical and horizontal pattern to thoroughly cover the plate.
4. After 5 min, the plate is again sprayed with 95% EtOH and wiped clean with LFTs. Repeat the 95% EtOH wash three more times. The latter washes remove excess bind silane that could dissolve in the gel and transfer to the opposite plate. While treating the adhesive plate, it is important not to handle the opposite, untreated glass plate with the same gloves because the bind silane is easily transferred. Gels cannot be removed intact if the nonadhesive plate becomes contaminated, even slightly, with bind silane.
5. The opposite plate is washed with a soft sponge and grease cutting dish detergent, rinsed in ddH_2O and once in 70% EtOH and then treated twice with 1 mL of Sigmacote (Sigma Chemical Co.) on a cotton gauze pad prior to each use.
6. To make 100 mL of gel solution, mix together 16.7 mL of a 30% acrylamide, 20 mL of 5× TBE, 58.3 mL of double glass distilled water (ddH_2O) and 5 mL of glycerol. Because glycerol is viscous, it is easiest to mix the first three components, draw the glycerol into a 5 mL pipette and allow it to drain into the stirring acrylamide solution. Immediately prior to pouring the gel, the gel solution is filtered in a Buchner funnel through three layers of filter paper (Whatman #1). Ammonium persulfate (100 μL) and TEMED (100 μL) are added and the gel is poured following manufacturer's instructions. Following polymerization, combs are removed and the wells are washed out with 1× TBE.
7. From each 50 μL PCR reaction, 5 μL is removed to a 500 μL tube and 1.5 μL of the appropriate loading buffer is added. The tube is tapped to mix the contents and spun down. For RAPDs, 3–4 μL (approximately 150 ng DNA) is loaded directly onto the gel. For SSCP analysis the mix is first heated to 95°C for 2 min and then plunged directly into ice.
8. Electrophoresis is performed at RT (20–26°C) for 16–24 h with a constant amperage of 16 mA on large (40 × 50 cm) gels. For smaller (20 × 20 cm) gels that can be chilled, electrophoresis is performed at 20 V/cm for 2–2.5 h at 20°C. Lower

voltages may be required in systems that cannot be chilled. For large gels, 1× TBE is used in both upper and lower buffer chambers while 0.5× TBE is used with small gels (Note 8 on differences between large and small gels).

31.3.5 Silver staining gels

All procedures are carried out with ddH_2O or 18.3 MΩ deionized ('Ultrapure') water to reduce dark and uneven background staining. For large gels, all staining procedures are carried out in 2 L, while for small gels 500 mL are used. Make up and chill (4°C) gel developing solution ahead of time. Chill the developing tray also.

1. Remove the glass plate with the adhered gel to 10% acetic acid in a shallow plastic tray and place it on a rotary shaker for 20 min. The gel can be stored indefinitely in fixative before proceeding with the next steps. Do not discard the fixative; it is used in terminating the developing reaction.
2. Rinse the gel three times in ultrapure water with 2 min of agitation per wash.
3. Transfer the gel to stain solution and agitate for 30 min. Remove the gel from the staining solution, drain and rinse it for no more than 20 s in fresh ddH_2O.
4. Place the gel in chilled developer (remember to add the formaldehyde and sodium thiosulfate immediately prior to use) and agitate rapidly by hand. As soon as bands become clearly resolved (usually 4–6 min), pour the fixative solution from the first step directly into the developer and agitate for 3 min.
5. Rinse the gel and plate twice in ultrapure water for up to 10 min and lean them against the wall to dry or photograph immediately (Note 9).

NOTES

1. Computer programs for the analysis of RAPD polymorphisms for population genetics and molecular taxonomy are available through electronic mail by contacting wcb4@lamar.colostate.edu. They are also available from the authors by sending two high density floppy disks. Please specify whether your computer has a math coprocessor.
2. RAPD polymorphisms have been criticized for lack of reproducibility among PCR runs made on the same organism, among individual members of a species and among labs (Black, 1993). This has been attributed to differences in the type of *Taq* polymerase used, concentrations of $MgCl_2$, ramp times among thermal cyclers, and the amount and quality of template DNA. The latter factor has been of special concern for those working with small insects in which the amount and quality of isolated DNA can vary. A single source of *Taq* polymerase should be adopted and a standard concentration of $MgCl_2$ should be maintained. A single brand or type of thermal cycler should be used for analysis of all individuals in a study. We have reduced the effects of variable amounts of DNA by increasing the concentration of primers to 1 μM so that primers essentially saturate the solution and anneal to all available template DNA. Degraded template DNA usually produces no fragments or a few small fragments. These template DNA preparations are discarded. A negative control is a tube containing all of the PCR reagents but without template DNA. It is handled simultaneously with all other

tubes. PCR runs that display contaminating bands in the negative control are discarded.

3. A good strategy for selecting RAPD primers useful in an insect species is to buy a kit of primers from a company, analyse these on several individuals from different populations and have large quantities made of the 4–5 primers that you identify as being useful. Operon Technologies Inc. (Alameda California) sells sets of 20 primers in quantities sufficient to do 50–100 test reactions.
4. For silver staining, water quality is essential to reduce background staining. We use either ddH_2O or 18 MΩ 'ultrapure' deionized water. A pair of polypropylene trays for large gels or glass dishes for small gels are used for silver staining. One holds the gel for fixing and staining while the other is used for washes and developing. It is critical that these trays be clean prior to and following the silver staining process to reduce uneven and background staining. Alconox works well. In addition, all reagents should be at least molecular biology grade or ultrapure quality. Use of less pure water and reagents produces gels with dark and uneven background staining.
5. The amount of template DNA needed to obtain optimal and consistent amplification of bands varies widely among vector species. A 10-fold serial dilution from 1/100–1/100 000 of an insect defines the optimal range of dilutions.
6. *Taq* polymerase is added at 80°C because some DNA isolation procedures generate single-stranded template allowing nonspecific primer annealing. *Taq* polymerase acts at RT and can extend the nonspecifically bound primer to cause artefacts that are subsequently amplified during PCR.
7. a. 50 pM of each primer flanking the targeted region are added.
   b. High annealing temperatures are used during amplification to maintain stringency with the specific primers. The initial denaturation and *Taq* loading conditions are as described in Section 31.3.2, step 5. A set of conditions that have proven useful over a wide variety of primers are:

Step 1: (denaturation)	92°C for 1 min
Step 2: (annealing)	48°C for 1 min
Step 3: (complete extension)	72°C for 2 min
Step 4: Cycle to step 1, 10 times	
Step 5: (denaturation)	92°C for 1 min
Step 6: (annealing)	54°C for 35 s
Step 7: (complete extension)	72°C for 2 min
Step 8: Cycle to step 5, 30 times	
Step 9: (final extension)	72°C for 7 min
Step 10: (overnight storage)	hold at 4°C

   Products are checked as described in steps 7 and 8 of that section. Consult Chapters 21 and 32 for further instructions on setting-up and running targeted PCR reactions.
8. There are many brands of vertical polyacrylamide gel electrophoresis boxes. For large gels we use the 38 × 50 cm Sequigen cell (Bio-Rad Laboratories, Hercules, CA). We use the Hoefer SE600 system (Hoefer Scientific Instruments, San Francisco, CA) to run smaller (14 × 16 cm) gels. Larger gels provide better separation of bands and give superior resolution of individual bands. They are, however, cumbersome and awkward to handle, score and photograph. They also consume more electrophoresis and staining reagents and can quickly become chipped with routine use.

9. About 4 h are required to isolate DNA from 50 individual specimens using either of the DNA isolation methods. DNA pellets are allowed to resuspend overnight. Once the DNA samples have been obtained, RAPD-PCR analysis requires 48 h to complete and SSCP analysis requires 24 h. Setting up 50 RAPD- or targeted PCR reactions requires about 2 h. The RAPD amplification program runs overnight; the targeted-PCR amplification program runs in 3–4 h. The agarose gels and polyacrylamide gels can be poured while the thermal cycler program is running. Large acrylamide gels can be set up and loaded in less than 2 h and are run overnight. Smaller gels can be run in 2–4 h. Gels can be fixed and stained within two hours or fixed and stored in the fixative at room temperature for several days.

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CHAPTER THIRTY TWO

PCR amplification of insect ribosomal DNA

Susan M. Paskewitz and Frank H. Collins

32.1 INTRODUCTION

Ribosomal DNA (rDNA) has been used extensively and very effectively for insect phylogenetic and population analysis. In this chapter, we will focus on its use in distinguishing between closely-related species. The rDNA genes encode the rRNA, which comprises three of the main RNA structural components of ribosomes. Because rRNA has a fundamental role in mRNA translation in all organisms, portions of the rDNA are highly conserved, being the same from bacteria to humans (Gerbi, 1986). These conserved portions provide sequences that can be used as primers to amplify regions of the rDNA by polymerase chain reaction (PCR) from organisms for which no prior DNA sequence information is known. The rDNA has other advantages as a target for systematics studies, including a variable rate of evolution across different sections and a generally high copy number within a genome.

The rDNA is usually arranged in tandemly linked genes, all arrayed in the same transcriptional orientation, with copy numbers ranging from 100 to 1000 in insects (Beckingham, 1982). The rDNA genes may occur in one long array or may be spread over several loci. Individual transcription units are separated from each other by intergenic spacer regions (IGS), also referred to as nontranscribed spacers (NTS). The IGS often contain short subrepeats, regions of which may be important in transcription, and these subrepeats may vary in number from one cistron to the next. Within each transcription unit, there are two major coding regions, for the 18S and 28S ribosomal RNA, and a region coding for the small 5.8S RNA which occurs within the internal transcribed spacer (ITS) region between the 18S and 28S subunits (Figure 32.1). The ITS is divided into the ITS1, lying between the 18S and 5.8S coding regions, and the ITS2, lying between the 5.8S and the 28S coding regions.

These different regions of the rDNA vary quite considerably and predictably in their levels of intraspecies and interspecies sequence polymorphism. For example, parts of the 5.8S, 18S and 28S subunits are highly conserved, with very little sequence variation detectable within an individual or species or between closely related species. The IGS regions, on the other hand, diverge quite rapidly even between very closely related species, and certain IGS sequences, particularly the number of

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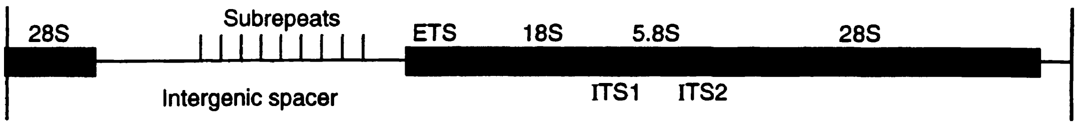


Figure 32.1 Structure of the ribosomal DNA. (ITS, internal transcribed spacer; ETS, external transcribed spacer.)

various subrepeats, tend to vary from one unit to the next within an individual organism. ITS1 and ITS2 sequences also experience relatively rapid rates of evolutionary divergence, although probably to a lesser degree than the IGS sequences. In general, ITS1 and ITS2 regions do not contain long subrepeat sequences that are variable intraspecifically (but see Paskewitz *et al.*, 1993a). Because these ITS are generally much shorter than the IGS sequences, they are more easily cloned and sequenced by strategies involving PCR. It should be noted, however, that some species may have levels of intraindividual and intraspecific variation in spacer sequences that can approach several percent, thereby complicating use of these sequences for species diagnostic purposes. These differing levels of sequence variability within and between taxa associated with different regions of the rDNA offer opportunities for using rDNA sequences to investigate questions at a variety of levels of phylogenetic interest.

Although many methods have been devised for identifying closely-related species, rDNA PCR diagnostics have several advantages. DNA-based identification techniques can be used on all stages and DNA is stable under many simple storage conditions (including ethanol preservation). Very small portions of the insect are required, leaving the remainder available for other types of analysis. Sufficient DNA is usually present in a few scales or a leg segment (Paskewitz *et al.*, 1993b), thus permitting a museum or even living specimen to be identified with minimal loss. Finally, rDNA PCR can be adapted for high throughput for use with large-scale field investigations. Since methods for using rDNA PCR to identify specific species are already available in the literature, e.g. *Anopheles gambiae* complex (Paskewitz and Collins, 1990; Scott *et al.*, 1993), *An. freeborni/hermsi* (Porter and Collins, 1991), *Culex* spp. (Crabtree *et al.*, 1995), *An. quadrimaculatus* complex, (Cornel *et al.*, 1995), this chapter will focus on outlining methods for finding diagnostic sequences in other groups and on designing the assay once these sequences are available.

32.1.1 PCR amplification of rDNA

The first step in devising an assay for identification of species by PCR amplification of rDNA is to identify sequences that differ between the species of choice. First, the genetic material used to search for these sequences must be unambiguously identified by some other method. Because both intra- and interindividual sequence differences may occur, several individuals from each species, preferably sampled from different populations, should be carefully examined in the initial screen. The exact numbers and population distributions necessary for these analyses will vary from species to species. Laboratory colony material may be adequate for preliminary experiments, but access to field populations will be needed for validation once a putative diagnostic set of primers is identified.

Templates are amplified using conserved primers. Highly conserved domains are found in the 18S, 5.8S, and 28S coding regions and these can be used as primers to amplify the ITS1 and ITS2 regions. Intergenic spacers may provide more variable sequences but may be difficult to amplify by PCR because of a combination of length variation and relatively long length. Recent advances in PCR technology, allowing amplification of longer stretches of DNA, may facilitate access to the IGS in some species. In addition, there are a number of highly divergent domains within the 28S coding regions that may have diverged sufficiently among the species of interest to be suitable targets for species diagnostic assays (e.g. Larson, 1991). After amplification, products are examined by agarose gel electrophoresis for length variation. If the species of interest display differently-sized target regions, then no further work other than validation of the sensitivity and specificity of the assay is necessary.

Usually, amplified ITS or 28S divergent domain regions from closely related species cannot be distinguished by size on agarose gels. Amplified templates are then sequenced or examined by restriction digest. Cloning may be necessary because many insects show nontrivial levels of intraindividual variation in ITS sequences (e.g. Crabtree *et al.*, 1995). Alignment pinpoints invariant sequences that differ between the species of interest.

32.1.2 Designing the species-diagnostic assay

Once invariant species-specific sequences have been identified, oligonucleotide primers can be designed. A simple method for distinguishing two species relies on a cocktail of three primers; one conserved primer anneals to an invariant sequence in both species and two diagnostic primers that anneal to species-specific sequences on the complementary strand. The diagnostic primers are selected so that one corresponds with a specific sequence from one species and the second, from a different location, corresponds to a specific sequence from the second species. The two diagnostic primers are designed in an orientation so that they will each amplify a fragment when combined with the conserved primer. Because each diagnostic primer amplifies a fragment of a different size, the identifying PCR products may be discriminated on agarose gels. This strategy can be extended to multiple species (Scott *et al.*, 1993). Additional conserved primers can be included if necessary. For example, Porter and Collins (1991) used both 28S and 5.8S conserved primers in conjunction with two species-specific primers to discriminate between *An. freeborni* and *An. hermsi.*

The assay will be more robust if multiple differences can be incorporated in the diagnostic primers but a single difference, placed at the 3′ end of the primer, can be sufficient. Certain nucleotides function better in this context. For example, 3′ T's have been shown to efficiently amplify, even when mismatched with G, C or T (Kwok *et al.*, 1990). Ideally, primers should be approximately 20 or more nucleotides long, depending on genome size and complexity but shorter primers have functioned well. Primers should be designed with similar GC profiles and without stretches of complementary nucleotides, especially at the 3′ ends. Once primers are designed and produced, empirical trials are necessary to determine the precise PCR parameters that will successfully amplify the discriminatory fragments without background. Because the primers are specific to the organisms and the rDNA templates are present in multiple copies, temperatures can usually be maintained at 45–50°C for annealing and 25–35 cycles should be sufficient. However, this format may have to

be altered for primers where 50% GC content cannot be maintained, or for shorter primers. Differences in melting temperature may be compensated for by increasing the concentration of the primer with the lower T_m (Scott *et al.*, 1993).

32.1.3 Validation of species-diagnostic assay

Once a diagnostic assay that discriminates between the specimens in hand has been developed, additional members of geographically distant populations and field collected-samples should be examined. If all members of these populations/samples (definitively identified to species by some other method) display the expected profiles, then the assay is ready for use with larger samples of field-collected material. Initial field trials compare the PCR diagnostic with another accepted method of discrimination for accuracy and reliability (Paskewitz *et al.*, 1993b; Taylor *et al.*, 1993). In general, each new geographical area where the method is used should first test primers against an accepted method of discrimination, since local or regional variation not detected previously may occur (Paskewitz *et al.*, 1993b).

32.1.4 Sensitivity and contamination

PCR diagnostic assays are extremely sensitive, with picogram amounts of DNA being sufficient for amplification. Because the assays are so sensitive, contamination with extraneous DNA should be avoided, although for most laboratories using rDNA PCR to identify mosquito species, contamination has not been a serious problem. This is probably due to the fact that many copies of rDNA occur in each mosquito genome, and these multiple copies of the template may sufficiently 'swamp out' any slight contamination.

Contamination can result from using equipment both for setting up reactions as well as for analysing them after amplification. Ideally, dedicated areas are used for each operation, and equipment, such as pipettors, is not shared between the areas. Additional preventive measures include the use of aerosol resistant tips to prevent aspiration of template or product into pipettors, aliquoting of PCR reagents, and the inclusion of negative controls in each run. When a large number of samples are to be examined, it is wise to include several negative controls, set up at intervals during the reaction preparation period. Negative controls contain all components of the reaction except for the template DNA, for which sterile distilled water may be substituted.

It is not possible to describe an rDNA-PCR protocol which will function for all insects under all conditions. What follows is an example of a procedure that has functioned well for identification and examination of the ITS2 from many species of Diptera. This protocol may have to be modified for other insect species; in particular, primer design and DNA extraction may need adjusting. It is recommended that the DNA sequence databases be consulted during the conserved primer design phase, since sequences from near relatives will enable the user to produce specific but not universal primers (thus reducing the possibility of amplification of contaminating DNA from fungal or bacterial sources). The following sample protocol is divided into two sections. First, the production of ITS2 sequences using conserved primers is described. Second, a brief description of the diagnostic assay for the *An. gambiae* complex is provided, along with notes concerning problems and solutions encountered during the use of this assay.

32.2 MATERIALS

32.2.1 Genomic (template) DNA preparation (Note 1)

1. Extraction buffer: 5.5% sucrose, 80 mM NaCl, 0.85 mM EDTA, 24 mM Tris base, 0.5% SDS, 80 mM Tris-HCl. Store at 4°C. Replace every 2–3 weeks.
2. 8 M potassium acetate. Store at RT.
3. 70% and absolute ethanol. Store below 0°C.

32.2.2 PCR amplification

1. Primers: Several aliquots should be prepared as freezer stocks at 100 μM in sterile distilled water. These can be stored at –80°C or –20°C but should not be subject to frequent freeze/thaw. Stocks can be further diluted for working aliquots which should also be stored frozen (Note 2). Sequences of conserved primers that have worked well for amplification of the ITS2 in several species of Diptera are provided in Table 32.1.
2. Reaction buffer: Reaction buffers are provided with the thermostable DNA polymerase enzymes. Buffers may, or may not, contain $MgCl_2$ and this should be carefully noted and the reaction conditions adjusted accordingly.
3. Thermostable DNA polymerase: Different types of enzyme may give different results under otherwise identical conditions. Not all thermostable DNA polymerases are licensed for PCR use.
4. $MgCl_2$ (25 mM stock): $MgCl_2$ can precipitate with repeated freezing and thawing and should be stored at 4°C.
5. Nucleotides: Stocks of 10 mM of dGTP, dATP, dCTP and dTTP.

32.3 METHODS

32.3.1 Amplification of ITS2 sequences using conserved primers

(a) Preparation of genomic DNA

1. Extract DNA from single individuals. Use methods for DNA extraction described in this manual (Chapter 14) or the following protocol (Note 3). In general, do not be concerned about shearing of DNA for PCR applications.

Table 32.1 Conserved primers for amplification of ITS2 from many Diptera

Name/function	*Oligonucleotide sequence (5′–3′)*
5.8S primer (ITS2)	TGTGAACTGCAGGACACAT
28S primer (ITS2)	TTGCTTAAATTTCAGGGGGT

2. Grind insects the size of a single mosquito in 100 μL extraction buffer in a 1.5 mL microfuge tube. Volumes can be scaled up or down for other insects. Grind specimen completely using Kontes blue plastic pestles or other brand fitted to microfuge tube (Note 4).
3. Incubate sample at 65°C for 30 min. Place in water bath as soon as sample has been triturated to inactivate DNA degrading enzymes.
4. Add 14 μL of 8 M potassium acetate (final concentration 1 M) to precipitate proteins. Mix well.
5. Incubate on ice 30 min.
6. Spin in cold microfuge at about 14 000 g for 10 min. Transfer supernatant to new tube.
7. Add 200 μL cold 100% ethanol to precipitate DNA. Mix well. Store at –80°C if necessary.
8. Centrifuge at 14 000 g for 20 min. Discard supernatant.
9. Rinse pellet in 100 μL cold 70% ethanol followed by 100 μL cold 100% ethanol. Invert on paper towel to dry. Air-dry 10–15 min.
10. Resuspend DNA to a concentration of approximately 2–4 ng/μL (e.g. about one female mosquito in 500 μL) in sterile double-distilled water (Notes 5, 6 and 12).

(b) Amplification and analysis of ITS2 regions

1. Set up master mix for PCR reactions. Each reaction contains 200 μM each deoxy nucleotide, 2 mM $MgCl_2$ (Note 7), 0.1 μM each primer (Table 32.1 for sequences; Note 8), 1× enzyme reaction buffer, 0.625 U thermostable DNA polymerase, and sterile water to give a total of 24 μL. The master mix contains enough of these reagents to aliquot 24 μL to each reaction of the experiment (Note 8).
2. Reactions should include trial templates, negative control and, if possible, a positive control (insect known to amplify with primers selected). Add 2–4 ng of template (1 μL) to 24 μL of the master mix. Overlay with sterile mineral oil.
3. Amplify. PCR conditions should be established for the specific equipment, primers and template DNA being used. The following profile has routinely worked well to amplify DNA from several mosquito species: 94°C for 5 min followed by 25 cycles of 50°C for 1 min, 72°C for 1 min, 94°C for 1 min. If possible, samples should be held at 4°C after amplification is complete.
4. Analyse 10 μL of the amplification products on 1% agarose/TBE gels containing ethidium bromide (add 2 μL of 10 mg/mL ethidium bromide to 100 mL warm agarose solution and pour; *ethidium bromide is toxic, handle it and gels with gloves, rinse all contacted labware*). Run DNA marker to estimate size and concentration of amplified product. Check for length differences of the amplified fragment between species of interest (it may be necessary to increase the percentage of agarose to detect small differences in length). If length differences occur, then further refinement of the assay is not necessary. (If amplification fails see Notes 2, 5, 10 and 11.)
5. Sequence amplified products. The PCR product can be directly sequenced by cycle sequencing. If direct sequencing reveals high levels of sequence variation, the PCR products should be cloned prior to sequencing. Cloning of PCR products is facilitated by the tendency of some thermostable DNA polymerases to add a single deoxyadenosine to the 3′ end of an amplified product. These 'errors' can be used in combination with vectors for PCR product cloning which

are designed with 3′ T overhangs to provide for reliable and efficient cloning. Alternatively, the PCR primers can be designed with restriction sites attached to the 5′ ends (usually flanked by a GC clamp to facilitate subsequent digestion). The products and vectors can then be digested with the restriction enzymes and ligated by traditional means.

6. Align sequences and identify species-specific variants.

32.3.2 Optimization of diagnostic assays

Selected species-specific primers should be evaluated and assays optimized against colony or field-collected material. Optimization of the assay can be accomplished by systematically varying the $MgCl_2$ (Note 7) and primer concentrations (Note 8), and by adjusting annealing and extension parameters. For longer fragments extension times may need to be longer (as much as 3 min per cycle; Taylor *et al.*, 1993). Annealing temperatures may need to be adjusted to accommodate primers of lower GC content. Temperatures between 42 and 55°C are most commonly used. In this range, lower temperatures may increase nonspecific amplification.

32.3.3 Example of rDNA-PCR diagnostic assay: identification of members of the *Anopheles gambiae* species complex

1. Extract template DNA as described above (see also Note 3).
2. Make a Master Mix containing all components except template in quantities sufficient for all reactions (Section 32.3.1b, step 1). For each 25 μL reaction, use 0.625 U Amplitaq polymerase, 2 mM $MgCl_2$, 1× reaction buffer, primers (Table 32.2 for sequences; Scott *et al.*, 1993) and sterile water to a total volume of 24 μL. Primer concentrations are 6.25 ng GA, 12.5 ng each of UN and MR, 18.75 ng of AR, and 25 ng of QD for each 25 μL reaction. Aliquot 24 μL of master mix to each reaction tube or well. Add 2-4 ng (1 μL) template to individual reaction tubes or wells. Include negative controls and positive controls (template from identified specimens of the *An. gambiae* complex). Overlay with sterile mineral oil.
3. Amplify: 30 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 30 s. Use of a thermal cycler that is adapted for 96 well microtitre plates will facilitate multiple identifications.

Table 32.2 Species-diagnostic primers (rDNA intergenic spacer sequences) for *Anopheles gambiae* complex. Adapted from Scott *et al.* (1993)

Name	*Primer sequence (5′–3′)*
UN	GTG TGC CCC TTC CTC GAT GT
GA	CTG GTT TGG TCG GCA CGT TT
ME	TGA CCA ACC CAC TCC CTT GA
AR	AAG TGT CCT TCT CCA TCC TA
QD	CAG ACC AAG ATG GTT AGT AT

4. Analyse on 2.5% agarose gel containing ethidium bromide (2 μL of 10μg/mL ethidium bromide per 100 mL agarose solution; toxic, Section 32.3.1b, step 4). Use DNA size standards to check quality of gel and electrophoresis conditions as well as to assign sizes to products.
5. Troubleshooting:
   a. For no bands in all lanes, check that ethidium was included in gel and see Notes 2, 5, 11 and 12.
   b. For missing bands in some lanes see Notes 5, 11 and 12.
   c. For smeared products see Notes 7 and 10.
   d. For additional bands see Notes 1, 4, 7 and 13.

NOTES

1. Be cautious of the source of water used for making solutions. Large plastic containers constantly refilled with double distilled water without cleaning may become coated with algae, which can provide contaminating templates for PCR. An alternative is to transfer water to glass containers that have been treated to destroy DNA or to purchase PCR-grade water from a reputable source.
2. Repeated freeze/thawing of primers or template may result in degradation.
3. Preparation of DNA for PCR can be simplified by grinding the insect in sterile water, boiling the sample for 10 min to inactivate enzymes that might act on DNA or polymerase enzymes, centrifugation for 5 min, and removal of the supernatant. An aliquot of the supernatant can then be used directly for PCR (Scott *et al.*, 1993). This protocol provides 'dirty' DNA that is not stable on freezing at –80°C but can be useful for high throughput identifications where the sample will not be needed for other applications.
4. Pestles should not be reused for PCR applications unless carefully treated with reagents known to degrade DNA, since small amounts of DNA may stick to the plastic and may not be destroyed during autoclaving.
5. The concentration of DNA in the template sample may vary widely and still provide successful amplification. In general, a lower concentration works better than a higher concentration. However, occasional specimens may have mostly degraded DNA and, in these cases, retesting a sample with increased template may yield an amplification product.
6. To obtain an estimate of the genomic DNA yield, an aliquot should be run in parallel with a known amount of DNA on an agarose gel. Compare the extent of ethidium bromide staining. As little as 1 ng or 1/100 of the DNA extracted from a mosquito leg have also been sufficient for amplification (Scott *et al.*, 1993; Paskewitz *et al.*, 1993a,b).
7. $MgCl_2$ concentrations for the reactions may be varied empirically between 1 mM and 4 mM. Concentrations higher than 4 mM usually result in increased non-specific amplification.
8. Making a master mix reduces problems due to inaccurate pipetting of small volumes. Master mixes should not be frozen and reused, since the enzyme is no longer protected from inactivation due to glycerol dilution. The reaction can be scaled up to provide additional DNA if needed.

9. Primer concentrations for the reactions may be varied empirically between 0.1 μM and 1 μM, but 12.5 ng of each primer/25 μL of PCR reaction (final concentration approximately 0.1 μM) has worked well in our hands (see also Note 2).
10. Smeared products may result from too much DNA polymerase. A master mix is recommended to avoid pipetting errors with this viscous enzyme.
11. Amplification of template DNA sometimes fails due to incomplete removal of SDS during the ethanol precipitation step of the DNA extraction procedure. Precipitate sample again using 1/10 vol 3 M sodium acetate and 2 vol ethanol and retest.
12. Storage of samples prior to PCR. Samples can be stored in 70% ethanol or isopropanol, frozen or carefully desiccated prior to use in rDNA PCR diagnostic assays. Batches of ethanol or isopropanol should be of good quality, without additives.
13. PCR products are usually easily detectable bands of an appropriate size. Some templates will also produce additional, fainter bands, which may correspond with the diagnostic bands from another species. In most cases, the strongest band represents the identifying band. If bands are of equal intensity they may represent a hybrid specimen, a contaminant problem, or intraspecific variation. Reamplify with fresh solutions.

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CHAPTER THIRTY THREE

Generation and use of species-specific DNA probes for insect vector identification

Julian M. Crampton and Susannah M. Hill

33.1 INTRODUCTION

Many insect vectors are members of complexes composed of morphologically identical sibling species. The identification of individual species, a requirement of epidemiological studies and control programmes, has traditionally relied upon techniques such as chromosomal analysis or isoenzyme typing. Due to the limitations of these techniques, the last few years have seen many developments in DNA-based technologies for identification. DNA-based protocols have advantages over the other techniques utilized in that they may identify all insect stages of both sexes using alcohol preserved, dried, fresh or frozen specimens. The methods ultimately rely upon either DNA probe hybridization or the polymerase chain reaction (PCR). This chapter describes the development and use of DNA probes for the identification of insect vector specimens. The aim has been to produce a methodology that is cheap, accurate and easy to use. In this chapter, the DNA probe-based techniques, which have been developed for the identification of *Anopheles gambiae* complex mosquitoes, are used to illustrate the power of these methods, although the technology is directly applicable to many other mosquito or insect vectors. In addition, the methods discussed may be utilized for generating additional epidemiological data such as identification of parasites within the vector or origin of the blood meal.

Mosquitoes of the *Anopheles gambiae* complex include the major vectors of malaria in tropical Africa. Six different sibling species within the complex have been identified on the basis of mating incompatibility (Davidson, 1964; Davidson and Hunt, 1973; Davidson *et al.*, 1967). None of them can be distinguished using morphological characteristics. *An. gambiae* sensu stricto and *An. arabiensis* are the two most efficient vectors within the complex and are widespread throughout tropical Africa, being sympatric over much of their range. *An. merus* and *An. melas*, two saltwater-associated forms, are vectors in coastal regions of East and West Africa respectively, while *An. bwambae* is a vector confined to the Semliki forest in Uganda. *An. quadriannulatus*

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is mainly zoophilic and therefore not considered to be a vector of malaria; it is found in limited locations in East and South Africa and Ethiopia.

The different species in the complex exhibit markedly different behaviour, ecology and vector capacity and respond differently to control measures. As two or three species are commonly found together in a given area, identification of the individual species is very important (Service, 1982). Currently, a number of methods are available for species identification including: polytene chromosome banding patterns (Coluzzi and Sabatini, 1967, 1968, 1969; Green, 1972), isoenzyme analysis (Mahon *et al.*, 1976; Miles, 1978, 1979), cuticular hydrocarbon determination (Carlson and Service, 1979; Hamilton and Service, 1983) and polymerase chain reaction (Chapter 31; Paskewitz and Collins, 1990; Paskewitz *et al.*, 1993). These techniques all require some expertise and expensive equipment to perform, or are limited by the life stage or means of preservation of specimens. They are therefore not entirely suitable for widespread field identification of large numbers of specimens. The use of DNA probes offers an attractive alternative to these techniques as protocols may be cheap, simple and quick to use and suitable for simultaneous identification of large numbers of specimens. Finally, DNA probes work on all insect stages and on alcohol preserved and dried material in addition to fresh and frozen mosquitoes.

A series of cloned DNA probes have been isolated from cloned genomic DNA libraries by differential screening which allows identification of five of the six species in the *Anopheles gambiae* complex (Gale and Crampton, 1987a, 1987b, 1988; Hill and Crampton, 1994b). *An. bwambae* was not included in the work because of its extremely localized distribution and lack of available material. The DNA probes are not ideal in that each probe does not uniquely distinguish a single species. Different degrees of hybridization are seen between each probe and the different species of the complex. These differences are consistent and allow specimens to be identified with the DNA probes using a key (Table 33.1).

The DNA probes have been used successfully in the laboratory for a number of years. However, several modifications have been made in order to make the DNA probes more suitable for use in the field. The major logistical problems for field use of DNA probes are the preparation of specimens, hybridization of the probe and the

Table 33.1 A DNA probe key to the *Anopheles gambiae* complex

Species		*pAnaI*	*pAnml4*	*pAngsI*	*pAnqI*	*pAngss*
An. gambiae s.s.	♀ and ♂	–	+	++	–	++
An. arabiensis	♀	–	+	++	–	–
An. arabiensis	♂	+++	+	++	–	–
An. melas	♀ and ♂	+	+++	++	+	++
An. merus	♀	+++	+++	++	+++	+
An. merus	♂	+++	+++	++	+++	++
An. quadriannulatus	♀	–	+	++	+++	+
An. quadriannulatus	♂	–	+	++	+++	++

(+++), A very strong positive signal; (++), a strong positive signal; (+), a weakly positive signal; (–), no detectable signal.

requirement for radiolabelling and subsequent associated hazards. These have been solved to a great extent by the use of mosquito squash-blots and nonradioactive oligonucleotide probes (Hill *et al.*, 1991a, 1991b), in conjunction with a simplified protocol requiring a minimum of equipment and expertise (Hill *et al.*, 1992).

The series of cloned DNA probes were sequenced in order to define the repetitive sequences that are species-specific. Sequences of 20–30 bases in length which appeared to be invariant within each species were used to prepare synthetic oligonucleotide probes to replace the cloned DNA probes (Table 33.2) (Hill and Crampton, 1994b; Hill *et al.*, 1991b). These oligonucleotide probes demonstrate the same hybridization characteristics as the original cloned DNA probes.

The oligonucleotides were labelled with the enzyme alkaline phosphatase using the E-link plus kit (Cambridge Research Biochemicals, Cheshire, UK). Oligonucleotides were first modified by incorporation of an N-TFA C_6 amino modifier (Cruachem, Sterling, VA, USA) as the final stage of synthesis. One tenth of the 0.2 μM product (100 μL) was conjugated with lyophilised, activated alkaline phosphatase and purified using the reagents, columns and instructions supplied with the kit. The alkaline phosphatase label could then be involved in a light-emitting chemiluminescent or a colorimetric detection reaction.

DNA probes have been isolated and used successfully in the laboratory for the identification of specimens of a number of important insect vector species, including *An. dirus* (Panyim *et al.*, 1988), *An. farauti* (Booth *et al.*, 1991; Hartas *et al.*, 1992), *An. quadrimaculatus* (Cockburn *et al.*, 1988), *Simulium damnosum* (Post and Crampton, 1988; Post and Flook, 1992) and *Phlebotomus papatasi* (Ready *et al.*, 1988). It is possible that any species-specific probes such as these could be developed for use in the field as nonradioactively labelled oligonucleotide probes with the simplified protocol. In addition, extra probes may be used in conjunction with vector species-specific probes to detect the presence of infecting parasites (Ready *et al.*, 1988; Delves *et al.*, 1989; Dissanayake and Piessens, 1992; Gibson *et al*, 1988). It is possible to use part of the body, such as the head, for DNA probe identification purposes leaving the remainder free for alternative analyses; for example ELISA analysis of the thorax for origin of the mosquito blood meal (Beier *et al.*, 1988), or presence of sporozoites (Wirtz *et al.*, 1987) and cytogenetic analyses using the abdomen (Graziosi *et al.*, 1990). The use of DNA-based methods for the identification of insect vectors and the detection of the pathogens which they transmit in the vector is reviewed by Hill and Crampton (1994a).

During the development of a DNA probe, the following points should be considered. The probe should allow discrimination between the insect vector DNA of interest and any parasites or other microorganisms which may be present, as well as other members of the same complex or different species. The probe must be sensitive enough to identify the insect from the small amount of target DNA present in a portion of the specimen.

The ideal candidate which fulfils both of these requirements is repetitive DNA (Jelinek and Schmid, 1982). These sequences are found in the spacer regions of the genome, and since they appear to lack sequence-specific functions tend to evolve rapidly. Consequently, different species may differ with respect to their repetitive DNA sequences. Repetitive DNA sequences have a high copy number, offering the sensitivity required for detection studies.

A third consideration in DNA probe development is that the potential probe must be made available in abundant supply. This is made possible by the use of DNA

Table 33.2 Synthetic DNA probes for the identification of *Anopheles gambiae* complex mosquitoes

Probe	*Species origin*	*Nature of tandem repeats*	*Length of repeats (bases)*	*Sequence of oligonucleotide*
pAngss	*An. gambiae* ss	Direct	68	5′-^{1}TAGA(T/G)TGTTTGTATGAACCTTGG^{23}-3′
pAngsl	*An. gambiae* ss	Direct	92	5′-^{1}TATCGTTGTTACGGCCATGTT^{21}-3′
pAnaI	*An. arabiensis*	Interspersed	41	5′-^{1}TGTTCAGAGTGAAGATATCTT^{21}-3′
pAnml4	*An. melas*	Direct	163	5′-^{1}CTAGGTATGTGCCCGTTCGTGACTAT^{26}-3′
pAnqI	*An. quadriannulatus*	Direct	30	5′-^{1}GCGACCGAA(C/A)A(C/A)TTTGCGACCAA^{23}-3′

cloning and/or *de novo* synthesis of oligonucleotide sequences. The latter allows detection tests to be both quick and simple.

The steps involved in DNA probe development and use are outlined below:

1. Isolate the insect DNA and clone it into a bacterial vector (Chapters 14 and 17).
2. Screen recombinant clones with labelled, total genomic DNA. This will preferentially identify clones containing repetitive sequences because of their high copy number (Chapter 17).
3. Check the specificity and sensitivity of the potential probe.
4. Sequence the cloned insert and identify the repetitive sequence of interest (Chapter 25).
5. Simplify the DNA probe by recloning the repetitive sequence or making a synthetic oligonucleotide of the repeated sequence.
6. Use the DNA probe with nonradioactive detection systems and squash–blot methods for insect specimen identification.

33.2 MATERIALS

33.2.1 Stock solutions

1. 10% SDS.
2. 1 M NaOH.
3. 3 M NaCl.
4. 1 M Tris-HCl pH 7.5.
5. 20×SSC (3 M NaCl, 0.3 M sodium citrate).
6. 1 M Tris-HCl pH 9.5.
7. 100 mM $MgCl_2$.
8. 50 mg/mL 5-Bromo-4-chloro-3-indolyl phosphate (BCIP, toluidine salt) in dimethyl formamide (DMF).
9. 75 mg/mL Nitroblue tetrazolium salt (NBT) in 70% DMF.

All solutions for squash–blots and nonradioactive DNA probe hybridizations may be made from the above stocks. Stock solutions 1–7 are made with general laboratory reagents from any suppliers, (i.e. BDH, Analar, Sigma, Fisons) dissolved in double distilled water. These stocks, and any solutions derived from them, may be stored for long periods at room temperature. BCIP and NBT are supplied by Boehringer Mannheim GmbH as solids; solutions are stored at 4°C in the dark.

33.2.2 Preparation of squash–blots

1. Whatman 3MM paper.
2. Hybond N^+ nylon membrane (Amersham, Bucks, UK).
3. Small glass plate.
4. Oven at 80°C.
5. Tissue preparation: 10% SDS.
6. DNA denaturation: 0.5 M NaOH, 1.5 M NaCl.
7. Filter neutralization: 0.5 M Tris-HCl, pH 7.5, 1.5 M NaCl.
8. DNA fixation: 0.4 M NaOH; 5×SSC.
9. Removal of debris: 0.1 M NaOH.

33.2.3 Hybridization of filters

1. Plastic bag sealer and plastic sheeting.
2. Filter wetting solution 1×SSC.
3. Prehybridization solution: 5×SSC, 0.5% SDS, 2% skimmed milk powder (may be any commercial brand (i.e. Marvel, Premier brands UK Ltd). Make up fresh solution as required.
4. Hybridization solution: prehybridization solution containing 1.0 nM labelled probe.
5. Nonradioactive oligonucleotide probes: Synthesized with sequences as published (Hill and Crampton, 1994a, 1994b; Hill *et al.*, 1991b) with an NTFA C_6 Amino modifier (Cruachem, Sterling, VA) at the 5′ terminus, and labelled using the E-Link plus™ system (Cambridge Research Biochemicals, UK)). Long-term storage of probes at –20°C in aliquots; do not refreeze thawed aliquots. The labelled probes are also stable at 4°C for at least 6 months.
6. Wash solution 1: 0.5×SSC, 0.5% SDS.
7. Wash solution 2: 0.1×SSC, 0.5% SDS.
8. Wash solution 3: 1×SSC.

33.2.4 Detection of hybridization

(a) Chemiluminescent detection

1. X-ray film (i.e. Fuji RX medical).
2. X-ray cassette (no intensifying screens).
3. Acetate sheets for overhead projector.
4. Lumiphos substrate (Cambridge Research Biochemicals, UK). Store at 4°C in dark. *Avoid inhalation and skin contact; use in a fume cupboard.*

(b) Colorimetric detection

1. Plastic bag sealer and plastic sheeting.
2. Colour reaction buffer: 100 mM NaCl, 1 mM $MgCl_2$, 50 mM Tris-HCl, pH 9.5.
3. Colour development buffer: 10 mL colour reaction buffer, 33 μL NBT stock, and 25 μL BCIP stock. Make up fresh immediately before use.

33.3 METHODS

33.3.1 Isolation of insect vector DNA and cloning in bacteria

The first step in the preparation of species-specific probes is to prepare a clone bank representative of the genomic DNA from the insect species of interest. The details of genomic DNA preparation and cloning are described in Chapters 14 and 17. It should be noted that as repetitive sequences are selected as species-specific DNA probes, a partial or 'incomplete' library is usually adequate for picking out potential candidate probes.

33.3.2 Screening of colonies for repetitive sequences

Colonies are transferred onto nitrocellulose, lysed *in situ* and the DNA fixed onto the support. The filters are hybridized in the presence of a denatured ^{32}P-labelled genomic DNA probe to pick out colonies containing repeated sequences. By screening the same colonies with genomic DNA probes from a number of species, species-specific probes can be identified. This process is illustrated in Figure 33.1.

33.3.3 Specificity and sensitivity of the DNA probe

The usefulness of the plasmid DNA as a probe for species determination needs to be assessed in terms of its sensitivity and specificity.

The probe, labelled with ^{32}P either by random priming or nick translation, must first be checked for cross-hybridization with other DNAs. 200 ng DNA from a range of sibling species and related insects are spotted onto nitrocellulose and hybridized to the probe under the optimal conditions determined (Chapter 20). If hybridization is detected, serial dilutions of DNA should be made to determine how sensitive the probe is to the cross-hybridizing DNA. If cross-hybridization is a problem, other potential probes must be sought.

To determine the sensitivity of a probe, dot–blots of genomic DNA are made over a range of concentrations (pg to ng) and hybridized to the plasmid. At this stage the optimal stringency conditions for washing filters can be determined. Squash–blots (Section 33.3.6) of whole specimens or portions of specimens can also be tested against the probe to determine the minimal and optimal amounts of material necessary to give a detectable signal.

33.3.4 Identification of repeated sequences

If the plasmid DNA probe is digested with a range of restriction enzymes, banding patterns may be obtained which are indicative of a repetitive sequence. If agarose gels of restriction digests are then Southern blotted (Chapter 20) and hybridized with ^{32}P-labelled total genomic DNA from the insect species of interest, the fragments containing repetitive DNA sequences will show intense hybridization signals with this labelled DNA. Confirmation and identity of the repeat sequence can be made by DNA sequencing (Chapter 25; Hill and Crampton, 1994a).

33.3.5 Simplification of the DNA probe

Once the repetitive sequence has been identified, the DNA probe can be simplified to reduce the likelihood of cross-hybridization. This is achieved by either subcloning the repeated unit, or synthesizing an oligonucleotide of the sequence repeat or the consensus sequence.

Ideally, oligonucleotide probes are usually between 17 and 30 nucleotides in length. When used as hybridization probes they offer the advantage of providing a simple and quick protocol. Specificity and sensitivity of the synthetic probe needs to be checked and optimal conditions established. Once the probe has been fully characterized it may used for specimen identification as described in the following sections.

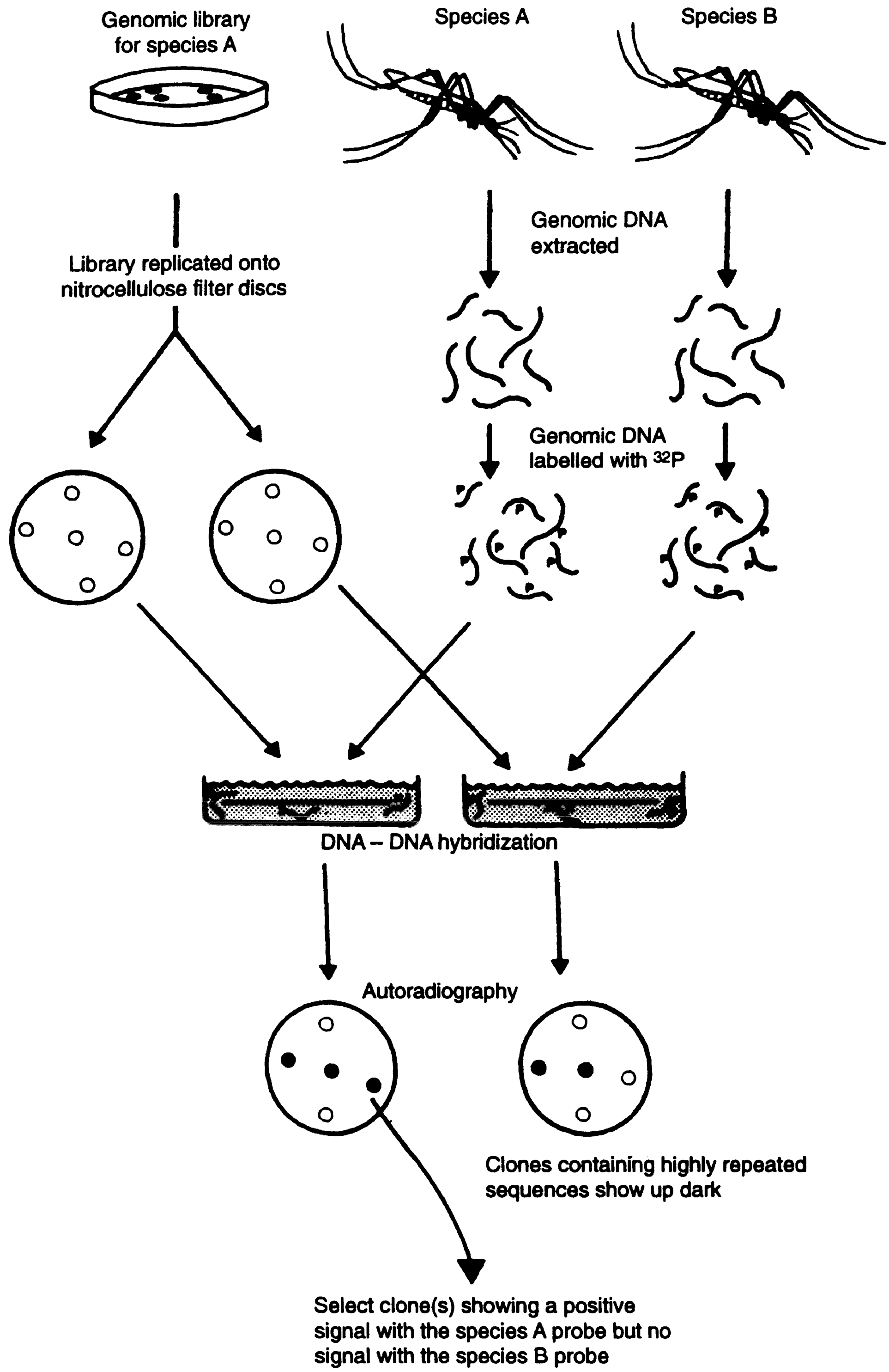


Figure 33.1 Selection of species-specific DNA probes by differential screening of an insect vector genomic library using labelled, total genomic DNA.

33.3.6 Squash–blot preparation

The following protocols are described using *An. gambiae* mosquito specimens as an example to illustrate the methods. Mosquito squash–blots provide a practical and simple method of preparing DNA from specimens for hybridization. Whole mosquitoes or individual portions, i.e. head, thorax or abdomen, can be applied directly to the nylon filter and lysed *in situ*. The recovery of DNA is very high and is comparable to that obtained by conventional extraction methods. The presence of impurities in the DNA does not affect hybridization with the alkaline phosphatase-labelled probes, but causes background problems with some other nonradioactive systems. The following method is derived from that of Tchen *et al.* (1985).

1. With a pencil, draw a 1 cm grid on nylon filter (Hybond N^+) allowing a single square for each specimen to be tested (Note 1). Cut the filter to the exact size required. Cut a second filter slightly larger than the first (Note 2).
2. Lay the nylon filters on Whatman 3MM paper saturated with 10% SDS (Note 3).
3. If individual portions of the mosquitoes are to be tested, dissect specimens with a clean scalpel wiping the blade with tissue following each dissection to prevent contamination of the subsequent sample (Notes 4–6).
4. Arrange the specimens on the surface of the first filter assigning one square of the grid to each sample (Note 7). Leave for 10 min for specimens to soak up the 10% SDS. The SDS softens the mosquito tissue.
5. To squash specimens, transfer the first, filter face-up, to a dry piece of Whatman 3MM and place the second filter on top (Note 8). Place a glass plate onto the filter sandwich and squash the specimens by 'thumping' the glass plate. The plate with the filters still attached may be turned over, and any specimens still intact squashed with the end of a glass rod.
6. Peel the filters apart with forceps to produce duplicate squash–blots (Notes 9 and 10).
7. Lay the filters face upwards onto Whatman 3MM soaked in 0.5 M NaOH, 1.5 M NaCl for 5 min to denature the DNA (Notes 10 and 11).
8. Transfer the filters onto 3MM soaked in 0.5 M Tris-HCl pH 7.5, 1.5 M NaCl for 5 min to neutralize the pH of the alkaline filter and DNA. During this procedure, remove chitinous body parts from the filter using forceps. Wipe the forceps with tissue after each specimen to prevent cross-contamination.
9. Place the filters on Whatman soaked in 0.4 M NaOH for 20 min to immobilize the DNA onto the filter (Note 12), then rinse the filters by immersion in 5×SSC for 30 s. Alternatively (and preferably) allow the filters to dry at RT then bake at 80°C for 2 h to fix the DNA.
10. Wash the filter in 0.1 M NaOH for 30 min with gentle agitation, then rub with gloved hands to remove any remaining material from the filter. Rinse the filter in distilled water. At this stage the filter may be dried and stored indefinitely or used directly.

33.3.7 Nonradioactive hybridization

This section describes the simplified protocol designed specifically for use in the field. Unlike other hybridization protocols, a complex hybridization solution and controlled or elevated temperatures are not needed, eliminating the need for expensive reagents and sophisticated laboratory equipment.

1. Make up the prehybridization solution fresh as required by the addition of water and 20×SSC to milk powder and shaking to create a cloudy suspension with no solid lumps of milk powder. Finally, the 10% SDS is added and mixed gently (Note 13).
2. Prewet the filter in filter wetting solution. From this point on the filter should remain wet (Notes 14 and 15).
3. Prehybridize the filter in prehybridization solution at RT for at least 2 h, with shaking if possible (Notes 16–18; for volumes see Note 13). Prehybridization blocks nonspecific binding of the probe DNA to the filter and DNA samples.
4. Transfer the filter to hybridization solution (Notes 16–19; for volumes see Note 13). Hybridize for 20 min at RT with shaking to allow binding of the single-stranded DNA probe to filter-bound homologous DNA.
5. Transfer the filter from hybridization solution to washing solution to remove unbound probe DNA. Perform the following washes at RT with shaking:
   Two, 5 min lower stringency washes in wash solution 1 (Notes 20 and 21);
   Two, 10 min higher stringency washes in wash solution 2 (Notes 20 and 21).
6. Finally, wash the filter for 5 min in wash solution 3 at RT to remove SDS from the solutions still present on the filter (Note 22). The filter may now be used in chemiluminescent (Section 33.3.8a) or colorimetric (Section 33.3.8b) detection reactions.

33.3.8 Detection of hybridization

Hybridization of alkaline phosphatase labelled DNA probes can be detected using one of two techniques. Positive results with chemiluminescent detection are observed due to blackening of X-ray film, whereas colorimetric detection results in the formation of a purple precipitate adhering to the filter at sites of hybridization. The chemiluminescent reaction is based upon the removal of a phosphate group from 'lumiphos' or AMPPD to form an unstable dioxetane intermediate. This then decays with the emission of light, detected by X-ray film. Colorimetric detection of hybridization is based upon the action of the alkaline phosphatase enzyme on the colourless, soluble BCIP and yellow, soluble NBT used as substrates. BCIP is oxidized to insoluble indigo following release of a phosphoryl group while NBT is reduced to diformazan, forming a purple precipitate.

(a) Chemiluminescent detection

1. Place the filter face-up on a clean acetate sheet in a fume cupboard and spray with lumiphos (Note 23).
2. Remove the filter and place it face-up on a second acetate sheet. Place another acetate sheet on top so that the filter is sandwiched between the sheets, then place face-up in an X-ray cassette. Wipe away any lumiphos leaking out from between the acetate sheets with a tissue.
3. In the dark, or under a safe light, place an X-ray film on top of the filter.
4. Incubate the X-ray cassette at 37°C for 1–2 h before development of the X-ray film and analysis of the results (Notes 24–27).

(b) Colorimetric detection

1. Make colour development buffer by adding BCIP and NBT to Colour Reaction Buffer immediately prior to colorimetric detection.

2. Place the filter in colour development buffer in a heat-sealable plastic bag or universal bottle and wrap in foil to exclude light.
3. Incubate at 37°C for the appropriate length of time (Note 25). Colorimetric results are directly visible, so that the progress of the reaction may be monitored at intervals and stopped when necessary by washing the filter in water for 10 min (Notes 26 and 27).

NOTES

1. Always handle nylon filter with gloved hands or with blunt-ended forceps, never with bare hands as this may cause background problems.
2. The second filter should be cut slightly larger than the first (i.e. about 0.5–1 cm longer and wider) to allow for some error in placement of the second filter directly on top of the first, prior to squashing.
3. All stages utilizing Whatman 3MM soaked in treatment solutions can be conveniently performed in plastic trays. The 3MM paper should be soaked but not swimming in solution as this can lead to smearing of the hybridization results. When laying nylon filters on 3MM saturated with the treatment solutions, ensure no air bubbles form between the paper and the nylon filter so that the filter wets evenly. This ensures each specimen on the nylon filter becomes thoroughly exposed to each solution in turn.
4. In addition to adult mosquitoes, individual larvae or pupae may also be squashed for testing, even the spermathecae of inseminated females have been squashed and used for the deduction of species identification from identity of the sperm DNA (Gale and Crampton, 1988).
5. It is not essential for specimens to be dissected before squashing. Sometimes the whole specimen may be necessary for a good result if spermathecae or early stage larvae are used or adult specimens of inferior quality.
6. In our experience, when mosquitoes are dissected into head, thorax and abdomen samples, squashed heads give the best results. This fortuitously allows alternative assays such as blood meal or cytogenetic analyses to be performed on the same specimens.
7. If possible, include control specimens from laboratory colonies of different *An. gambiae* species with identities confirmed by an alternative technique, such as cytotaxonomy.
8. Pick up the filters with two pairs of forceps from the centre of two opposite edges allowing the filter to bow slightly to prevent samples falling off.
9. It is important to note that the second squash–blot is a mirror image of the first. The second filter, known as the 'duplicate', is recognizable as not being marked with a grid. It is beneficial to make a pencil mark on each of the filters at this stage as a reminder of the alignment of specimens on the first filter and its mirror image on the duplicate.
10. Do not be alarmed if following squashing or during denaturation or neutralization, leakage of a large amount of greenish (from mosquito bodies) or reddish brown (from mosquito heads) pigment is observed surrounding the specimens. This pigment does not appear to affect subsequent results.
11. Double-stranded DNA is made single-stranded during alkali denaturation. This allows the probe DNA with a complementary sequence to bind with the single-stranded target DNA to reform a double-stranded DNA molecule.

12. The nylon filter possesses a positive charge which binds the DNA due to an electrostatic interaction. Treatment with sodium hydroxide should render this binding irreversible. Sodium hydroxide treatment may vary from 2–30 min; 20 min is used routinely for convenience. Although there appears to be little difference in efficiency of DNA fixation by alkali treatment or baking with Southern or DNA dot–blots, with squash–blots baking is far superior. It is recommended therefore that if a baking oven is available, this alternative method is used.
13. When making up prehybridization solution, the suspension should not be shaken once SDS is added as frothing occurs. This will create problems if plastic bags are used for prehybridizations. The volume of solution required for the prehybridization and hybridization steps depends on the size of the filter. For prehybridization, 500 μL–1 mL cm^{-2} of nylon is sufficient; for hybridization, 250–500 μL cm^{-2} may be used. For filters of average size (e.g. 10 cm^2, which allows for 100 samples), 20 mL of prehybridization solution is commonly made up in 20 mL graduated universal bottles. To 0.4 g of milk powder, 5 mL of 20×SSC and 14 mL of distilled water are added using the graduations marked on the side of the bottle. Vigorous shaking (with a secured lid!) creates a cloudy suspension to which 1 mL of 10% SDS may be added. Ten mL of this solution may be used for prehybridization, while the remaining 10 mL is retained for two 5 mL hybridization volumes.
14. Filter wetting and subsequent washing is best performed in approximately 100–200 mL of solution (depending upon the size of the filter and container) in small plastic sandwich boxes.
15. Drying out of the filter creates background problems following detection and care should therefore be taken transferring the filter between containers and solutions. In addition, serious background problems occur if the filter is not prewetted in 1×SSC.
16. Prehybridization and hybridization are performed preferably in heat-sealable plastic bags, though universal bottles, capped centrifuge tubes or sandwich boxes may be used. Use of sandwich boxes usually increases the amount of probe needed for each hybridization, which substantially increases the cost of the procedure.
17. Ensure that filters are completely wetted by the prehybridization and hybridization solutions. It is possible to treat more than one filter at once in the same solution by placing them apart and/or back-to-back. Care must be taken to ensure multiple filters do not stick together. When using heat-sealable plastic bags it is important that large air bubbles are removed to ensure an even coating of solution and prevent spotty backgrounds. Shaking is desirable also to reduce this effect. If using bottles or tubes, the filter should be rolled up inside and the tube frequently rotated if the solution does not cover the filter.
18. Prehybridization can be carried out overnight if more convenient. This allows more time the subsequent day for hybridization, washing and detection.
19. Care must be taken when handling the DNA probes to avoid nuclease contamination which will rapidly destroy probe activity. Use of gloved hands and sterilized pipette tips generally avoids this, although aliquotting of probe stocks and sequential use of individual aliquots will prevent loss of a large amount of probe DNA if nuclease contamination occurs.
20. Stringency of washing determines the degree of homology required for probe DNA to remain hybridized and may be altered by a change in temperature or salt concentration of the wash solution. Increasing the stringency (an increase in

temperature and/or a decrease in salt concentration) of washes increases the required homology. As washes here are performed at RT, a change of salt concentration is used to control stringency.

21. The washing stringencies have been determined as those most suitable for use with the probes for identification of *An. gambiae* complex mosquitoes. If this system is utilized with alternative oligonucleotide probes for other identification purposes, different washing conditions may need to be determined.
22. The presence of SDS inhibits the activity of the alkaline phosphatase enzyme attached to the probe. Activity of the enzyme is required for both chemiluminescent and colorimetric detection reactions.
23. Lumiphos for chemiluminescent detection should be used as sparingly as possible. Usually one or two 'sprays' are sufficient for a filter of 100 specimens. Using a pair of forceps the filter may be picked up and turned over to 'mop up' any excess lumiphos spray on the acetate. *Care must be taken to ensure the lumiphos is not inhaled or brought into contact with skin, therefore a fume cupboard should be used if available; if not, a mask may suffice.*
24. Chemiluminescent detection reactions may need different times of exposure varying from as little as 10 min to as long as 5–6 h. If exposure time is found to be insufficient or too long following development of the X-ray film, the filter may be re-exposed. Re-exposure is possible up to several days after the detection reaction as the light-emitting reaction is prolonged and still detectable after a week. Overexposure results in an increase in background in relation to positives.
25. Chemiluminescent and colorimetric reactions may be incubated at RT if a 37°C incubator is not available or a long exposure is required. An overnight incubation at RT is equivalent to 2–3 h at 37°C. This is because the alkaline phosphatase enzyme reaction is temperature dependent.
26. Two or more probes may be needed to identify an individual specimen conclusively. The duplicate filter may be used for hybridization of a second probe, if a third hybridization is required the filters may be stripped using 1% SDS at 80°C for 20–30 min and then reprobed. For filters used in colorimetric detection, the colour must first be removed by incubation in DMF at 60°C until all the colour has been removed from the filter.
27. Once the extent of different probe hybridizations with a mosquito specimen have been scored, the species may be deduced from the identification key shown (Table 33.1). It is important when using the key to compare degrees of hybridization with a standard for each set of samples. This is because results have been observed to vary considerably depending on factors such as amount of specimen used for identification purposes, the method and duration of storage. The complex-specific oligonucleotide, pAngsl, in addition to determining that samples collected belong to the *An. gambiae* complex, provides a rapid assessment of the quantity of DNA from each specimen available for identification. This is because pAngsl effectively hybridizes with equal intensity to DNA from all species in the *An. gambiae* complex tested.

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CHAPTER THIRTY FOUR

Cloning and detection of insecticide resistance genes

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34.1 INTRODUCTION

Insecticides act in impairing the function of molecular targets, usually proteins, which are crucial for life, and most resistances develop through the selection of mutations which either modify the target so that it remains at least partially functional, or increase the rate of insecticide degradation so that less toxicant reaches the target. Other resistance mechanisms include behavioural avoidance of the insecticide, decreased penetration and increased excretion; the first two mechanisms have been described in insects.

For each type of insecticide, one or several of these mechanisms can be selected in any given species. At present, only a few resistance genes have been cloned and sequenced in a small number of species, and our understanding of the different resistance mechanisms is still too incomplete to generalize the results to all resistance genes and to all species. Thus, between the first sign of resistance to an insecticide (revealed by bioassays) and the cloning of the gene(s) involved, a long series of complementary experiments must be undertaken.

In this introduction we will first give general information on the work which has to be performed before cloning can be envisaged. We will then briefly outline the different strategies which can be undertaken for cloning resistance genes and for identifying the mutations which are responsible for resistance. In many cases these strategies use techniques which have been described in other chapters of this manual and only the steps which are peculiar to resistance genes will be developed.

Although detection of resistance genes can be done by using nucleic acid probes, it can also be performed by using antibodies or/and by analysing the function of the proteins encoded by resistance genes (enzymatic activity or inhibition characteristics, ion permeability, etc.). This explains why we have developed the techniques to purify proteins and study their properties.

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34.1.1 Identifying resistance mechanisms and resistance genes

Investigations on resistance require a comparison of susceptible and resistant insects. Since field populations usually contain a mixture of susceptible and resistant genotypes (either homozygous or heterozygous), the first step is to obtain homozygous strains. Susceptible strains are usually available from already established laboratory colonies. Selection of a homozygous resistant strain involves exposure to insecticide doses every generation, until the dose–mortality line established by bioassays is stabilized. Homozygosity of resistance can be verified by bioassaying the F1 offspring of crosses between susceptible and resistant parents; the dose–mortality curve should be linear and parallel to that of one of the parents. Such a cross will show also whether resistance is dominant or recessive and will help in planning further selection.

The second step is to determine whether the observed resistance is controlled by one, or several genes. For diploid vectors with a sexual reproduction, the segregation of resistance will be studied on the offspring of back-crosses between F1 and the appropriate parental strain. If resistance is due to a single major gene, or to closely linked genes, the dose–mortality line of the back-cross will display a plateau at 50% mortality or an inflexion. Studying the linkage relationship between resistance and other genetic markers (morphological mutants, allozymes, microsatellites, etc.) will give valuable information when the cloning procedure(s) of the resistance gene(s) are chosen (Table 34.1 for abbreviations).

Table 34.1 Insecticide and enzyme abbreviations used in the text

Abbreviations	*Full name*	*Note*
Insecticides		
OP	Organophosphates	
CB	Carbamates	
PY	Pyrethroids	
CY	Cyclodienes	
AV	Avermectin	
Bt	*Bacillus thuringiensis* toxins	
Bs	*Bacillus sphaericus* toxins	
Enzymes and proteins		
AChE	Acetylcholinesterase	Target of OP and CB
Na + Vdp	Na + voltage-dependent channel	Target of PY and DDT
GABA receptor	GABA receptor of chloride channel	Target of CY and AV
Esterases	General esterases	Detoxifying enzymes
MCE	Malathion-carboxylesterases	Detoxifying enzymes
GST	Gluthatione *S*-transferases	Detoxifying enzymes
MFO	Mixed function oxidases	Detoxifying enzymes
P450	Cytochromes P450	Detoxifying MFO

A first indication of the nature of resistance genes involves testing cross-resistances between insecticides of different families, and/or comparing how the insecticide tolerance of susceptible and resistant strains is affected when bioassays are performed in the presence of synergists. Cross-resistance between carbamate (CB) and organophosphorous (OP) insecticides suggests that at least part of the resistance is likely to be due to an insensitivity of the acetylcholinesterase (AChE) target, whereas cross-resistance between pyrethroids (PY) and DDT corresponds probably to a decreased sensitivity of the Na^+voltage-dependent (Na^+Vdp) channel target. Modifications of resistance ratio by the synergist S,S,S-tributylphosphotrithioate (DEF) point to an increased detoxification by esterases and/or, in some cases, by glutathione S-transferases (GST) and modifications by the synergist piperonyl butoxide (PB) to an increased detoxification by mixed function oxidases (MFO) including cytochrome P450.

The next steps involve specific biochemical and/or physiological investigations comparing susceptible and resistant strains. Insensitivity of AChE will be revealed by comparing inhibition constants (k_i) of AChE in the presence of various OP (oxidized forms) and CB. Insensitivity of the Na^+Vdp channel is analysed by comparing the binding affinity of various toxins in the presence or absence of DDT or PY. Insensitivity of the receptor of *Bacillus sphaericus* (Bs) or *B. thuringiensis* (Bt) toxins is investigated by analysing the binding affinity of proteins of the gut brush border cells with ^{125}I-labelled toxins. Resistance to cyclodienes (CY) due to a modification of the GABA receptor of chloride-ion channel can be investigated by patch-clamp electrophysiology. Increased activity of detoxifying enzymes can be studied in comparing the *in vitro* and/or *in vivo* metabolization of ^{14}C-insecticides, or in measuring the activity of esterases, GST or MFO by biochemical assays using appropriate chromogenic substrates or, in the case of cytochrome P450, by analysing the absorbence spectrum. In all cases, it will be necessary to verify that the biochemical or physiological differences found between susceptible and resistant insects are responsible for the observed resistance. One possibility is to show that these characteristics remain strictly associated in the offspring of appropriate back-crosses. However, note that such an association may be observed if the two characters are tightly linked.

34.1.2 Strategies for cloning resistance genes

Once the resistance mechanisms have been identified, various strategies for cloning the structural genes encoding the proteins involved in resistance are possible.

Several of the proteins involved in resistance to insecticides have been cloned and sequenced in other organisms. When these proteins are coded by DNA containing conserved nucleotide sequences, these sequences can be identified and used to design primers which will allow amplification of genomic DNA or cDNA by PCR. The DNA fragments thus obtained can be used to screen DNA libraries. In other cases, it is necessary to purify the protein, either to obtain specific antibodies and use them to screen a cDNA expression library (as was done for example for *Culex pipiens* esterase B, Mouchès *et al.*, 1986), or to sequence peptides and design oligonucleotides for PCR (Franciosa, 1994). Another strategy, used by Hall and Spierer (1986) for cloning AChE and by ffrench-Constant *et al.* (1990) for cloning the GABA receptor of the GABA-gated chloride ion channel, was chromosome walking. At present, such a chromosome walk can only be envisaged in *Drosophila*, and possibly in

An. gambiae, due to the lack of a sufficient number of genetic markers in other insects. It must be noted that when the resistance mechanisms are not well characterized, it may be easier to first clone the homologous genes in *Drosophila,* which is genetically well known, and then use the sequence homologies to clone the same gene in another species. In the past, this strategy was used successfully for cloning AChE and the GABA receptor in various species including mosquitoes (Hall and Malcolm, 1991; Thompson *et al.*, 1993).

34.1.3 Identification of the mutations responsible for insecticide resistance

Once a structural gene has been cloned, the nature of the mutation(s) conferring resistance has to be identified. At present, the mutations which have been found in resistant insects include nucleotide substitutions and/or increased production of mRNA sometimes associated with gene amplification.

Point mutations are identified by comparing the sequence of the genes present in susceptible and in resistant insects. These mutations can then be produced, independently or in combination, by site-directed mutagenesis of a susceptible cDNA which can be expressed in a heterologous system such as *Xenopus* oocytes. The characteristics of the protein thus produced can then be studied either by the physiological characteristics they confer to these oocytes (as done for the GABA receptor by ffrench-Constant *et al.*, 1993), or by analysing their kinetic properties (as done for AChE by Mutéro *et al.*, 1994).

Quantitative variations of DNA and/or mRNA must be associated with the over-production of the concerned enzyme, and with the physiological expression of resistance. Again this can be done by verifying that resistance and larger quantities of RNA or DNA are always genetically linked. Other verifications can be devised. For example, to demonstrate that over-production of an esterase able to sequester insecticide was a resistance mechanism, Hoffman *et al.* (1992) used transgenesis to experimentally increase the number of gene copies of a susceptible AChE in *Drosophila.* They showed that an increased amount of AChE, an enzyme which irreversibly binds to OPs, induced a significant resistance.

34.2 MATERIALS

34.2.1 Assays to study enzyme activities

(a) AChE activity and inhibition

1. SP buffer: 0.1 M sodium phosphate, 1% Triton X-100, pH 7.8.
2. DTNB solution: 36.6 mg 5-5-dithiobis-2-nitrobenzoic acid (Sigma no. D8130), 15 mg of $NaHCO_3$ in 10 mL H_2O.
3. Insecticide stock solutions: serial solutions from 1 M to 10^{-5} M in ethanol. Just before use, prepare insecticide work solutions by diluting each ethanol solution in SP buffer or in water (1:99, v/v). Ethanol stock solutions are stored at 4°C.
4. Stain solution: 5 mL SP buffer, 0.5 mL 0.1 M acetylthiocholine iodide (Sigma no. A5771), 1.25 mL of DTNB solution, complete with H_2O to 50 mL. Prepare daily and keep in dark.

(b) General esterase activity

1. Sodium phosphate-Triton: 0.1 M sodium phosphate, 0.5% Triton X-100 (w/v), pH 6.5.
2. Alpha-naphthyl acetate stock solution: 20 mM in ethanol. Store at 4°C.
3. Substrate solution: 1 mL alpha-naphthyl acetate stock solution and 24 mL sodium phosphate-Triton. Prepare daily.
4. Stain solution: 37.4 mg Fast Garnett GBC in 25 mL sodium phosphate-Triton. Prepare daily.
5. Naphthol standard solutions: 6 solutions (3.0, 2.5, 2.0, 1.5, 1.0 and 0.5 M) of alpha-naphthol in ethanol.
6. Alpha/beta-naphthyl acetate: 1 g alpha-naphthyl acetate, 1 g beta-naphthyl acetate, bring to 100 mL with acetone. Store at 4°C.

(c) MCE activity

1. ADH: alcohol dehydrogenase (Boehringer no. 127.400).
2. NADH-diaphorase (Sigma no. D3752).
3. INT: *p*-iodonitrophenyl tetrazolium.
4. Malathion: 150 mM in acetone.
5. PMS: phenazine methosulfate; NBT: nitroblue tetrazolium.

(d) GST activity

1. DCNB: 10 mM dichloronitrobenzene in ethanol.
2. CDNB: 10 mM chlorodinitrobenzene in ethanol. Ethanolic DCNB and CDNB solutions can be kept a long time at 4°C in the dark.
3. GSH: 10 mM reduced glutathione in water. Prepare freshly as needed.

(e) MFO activity and P450 content

1. PMSF: 28 mg of phenylmethane sulfonate/mL of methanol.
2. PMSF-phosphate buffer: 400 mL sodium phosphate 0.5 M pH 7.2 with 1 mL PMSF.
3. PMSF-phosphate/glycerol: PMSF-phosphate buffer containing 20% glycerol (if samples are used immediately or kept at –20°C) or 30% glycerol (if samples are kept at –80°C).
4. 7-Hydroxycoumarin (0.01 mg/mL ethyl acetate).
5. 7-Ethoxycoumarin (20 mM in methanol).
6. NADPH (40 mM in water).
7. PMSF-phosphate/ethanol: PMSF-phosphate buffer and ethanol (v:v).

34.2.2 Purification of proteins

(a) AChE purification

1. Affinity chromatography column preparation: dissolve 0.124 g EEDQ (N ethoxy-carbonyl 2 ethoxy 1,2 dihydroquinoline) in 10 mL ethanol, dissolve 1.36 g procainamide in 10 mL water.

2. Mix EEDQ and procainamide solutions to 20 mL ECH Sepharose 4B (Pharmacia) by inversion and keep at 4°C overnight with shaking (magnetic stirring should not be used).
3. Pour the gel in a column, and wash it successively with 50% ethanol, with 20 mM Tris-HCl pH 7 (to block any remaining active group), and with 1 M NaCl. Equilibrate in 20 mM Tris-HCl, pH 7, 0.1% Triton X-100. The affinity column may be used several times and may be washed with water followed by 0.1% SDS to avoid eventual sample contaminations.

(b) Over-produced esterases purification

1. Imidazole: 0.025 M imidazole adjusted at pH 7.4 with HCl (1 N) (can be kept several weeks at 4°C).
2. Pharmacia polybuffer: prepare as indicated by Pharmacia to get pH 7.4, but dilute 10× instead of 8 (it is very important to degas this solution).
3. ^{14}C-DFP: ^{14}C-diisopropylfluorophosphate.
4. Laemmli buffer: 0.1 M Tris-HCl, pH 6.8 (31.2 mL), 10% SDS in water (10 mL), β-mercaptoethanol (2.5 mL), glycerol (5 mL), bromophenol blue.

(c) MCE purification

1. Preparation of gel filtration column: Fill a 15 × 900 mm column with superfine Sephadex G200. Check the gel packaging using Dextran Blue (T2000; PM = 2000 kDa). Equilibrate the gel with 10 mM Tris-HCl, pH 8.0.
2. Preparation of ion exchange chromatography column: Fill a 10 × 500 mm column with DEAE Fractogel. Equilibrate gel with 200 mL of 10 mM Tris-HCl, pH 8.0.
3. Tris-saline: add 36 g of NaCl to 1 L of 10 mM Tris-HCl, pH 8.0.
4. Preparation of hydroxyapatite chromatography column: fill a 10 × 150 mm column with a hydroxyapatite gel (Bio-Gel HTP, Biorad). Equilibrate gel with 10 mM sodium phosphate, pH 6.8.
5. Tris-glycine: 0.1 M Tris. Add glycine until pH 8.3 is reached. Add glycerol (10% w/v).

(d) GST purification

1. Glutathione-agarose column. Mix the glutathione-agarose powder (Glutathione linked to agarose *via* amino group, Sigma no. G9761) with distilled water (1/10; w/v) and let it stand for several hours at 4°C. Pour the gel in a 10 × 1 cm column with appropriate adaptators and wash it with 0.02 M sodium phosphate, pH 6.9.
2. Tris-GSH: 50 mM Tris-HCl, pH 9.5, 7 mM GSH (= reduced glutathione).

34.2.3 Other materials

(a) RACE for cloning resistance genes

1. Guanidinium buffer: 76.3 g guanidinium HCl (8 M), 0.39 g MES (20 mM), 4.0 mL 0.5 M EDTA, 0.347 mL 50 mM β-mercaptoethanol, H_2O to 100 mL.

(b) Dot–blot immunoassay for quantifying proteins

1. Tris-saline (TS) buffer: 0.5 M Tris , 2 M NaCl. Dissolve Tris in about 800 mL H_2O; adjust to pH 7.4 with HCl; add NaCl and complete volume to 1000 mL with H_2O. Dilute 10×.
2. TS-Triton buffer: 0.2% Triton X-100 and 0.2% SDS in TS buffer.
3. AP buffer (alkaline phosphatase buffer): 0.1 M Tris, 0.1 M NaCl, 5 mM $MgCl_2$; adjust to pH 9.5 with HCl.
4. TS-SBA: 3% bovine serum albumin in TS buffer.
5. TS-SBA/antiserum: 7.5 mL TS-SBA and 0.037 mL of antiserum.
6. TS-SBA/AP: 15 mL TS-SBA and 0.0021 mL of secondary antibody conjugated with alkaline phosphatase.
7. AP stain: 15 mL of AP buffer, 0.033 mL of nitroblue tetrazolium solution (50 mg/mL) and 0.0165 mL of 5-bromo-4-chloro-3-indoyl phosphate solution (50 mg/mL).
8. Tris-EDTA: Tris (0.2 M), EDTA (5 mM) adjusted to pH 8.0 with HCl.

(c) TEP test for detecting AChE genotypes in single insects

1. SP buffer (Section 34.2.1a).
2. 0.001 M propoxur in ethanol: prepared daily from a 0.1 M solution in ethanol by diluting in water.
3. Stain solution (prepare daily and keep in the dark): 1 mL of SP buffer, 40 mg of acetylthiocholine, 20 mg of DTNB (5-5-dithiobis-2-nitrobenzoic acid). This is the quantity of stain solution needed for one microtitreplate.

(d) Detecting esterase B gene amplification in single insects

1. Buffer A: 1% SDS, 50 mM Tris-HCl, 25 mM NaCl, 25 mM EDTA, pH 8.0.
2. Buffer B: 3 M potassium acetate, pH 7.2.

34.3 METHODS

34.3.1 Biochemical characterization of resistance mechanisms and genes

This chapter describes the biochemical assays used in our laboratories to measure the catalytic activity of enzymes known to be involved in insecticide resistance. One should keep in mind that there are large variations in the kinetics of these enzymes and care should be taken to adapt these methods in order to measure the highest activity.

The target proteins of insecticides which cannot be studied by their catalytic activity will not be considered here and readers will have to consult specialized publications. They include: the Na^+Vdp channel, target of DDT and PY (Pauron *et al.*, 1989), the GABA receptor of chloride ion channel, target of CY and avermectins (ffrench-Constant *et al.*, 1993), and the receptor proteins of the midgut brush border membrane, targets of Bs and Bt toxins (Nielsen-Leroux and Charles, 1992).

(a) Activity and inhibition of AChE

The main characteristic of resistance to OP and CB due to AChE is a decreased inhibition of the enzyme by some of these compounds. Usually, there is a good correla-

tion between the degree of AChE insensitivity and the resistance ratio observed in bioassays with any particular insecticide. Note that for OP, only the oxidized ('oxon') form inhibits AChE. For *Culex pipiens* AChE the carbamate propoxur is often used. (Solutions are listed in Section 34.2.1a.)

1. Insect homogenate: Grind insects in a microfuge tube in SP buffer (0.25 mL per mosquito). Centrifuge 5 min at 5000 g, and let stand 30 min at RT.
2. To compare residual activity of susceptible and resistant insects: Introduce in each cuvette 0.1 mL homogenate, add 0.01 mL insecticide work solution; let stand 15 min at RT; add 0.89 mL stain solution and mix rapidly; wait 3 min and measure the optical density of A_{412}. In blank, homogenate is replaced by 0.1 mL SP buffer, and in control insecticide work solution is replaced by 0.01 mL water. For each assay calculate D_{OD}/min. Residual activity at each insecticide concentration is calculated as:

$$(D_{OD}\text{insecticide} - D_{OD}\text{blank})/(D_{OD}\text{control} - D_{OD}\text{blank})$$

3. To measure k_i: Mix 0.9 mL homogenate and 0.1 mL of insecticide work solution of a given concentration. At times 1 min, 5 min, 10 min, 20 min, 30 min and 50 min, take 0.1 mL of the mixture, add 0.9 mL of stain solution and measure OD as previously. To calculate k_i, plot the relation between ln of residual activity as a function of time. A straight line is obtained, and its slope corresponds to $k_i \times (I)$, (I) being the insecticide concentration. Several estimates of k_i with different insecticide concentrations should be made to ensure that the conditions correspond to a pseudo first order kinetics; k_i is expressed in min^{-1}, mol^{-1}.

(b) Activity of general esterases

These esterases may display an increased activity toward synthetic substrates such as esters of naphthol, 4-methyl-umbelliferol or *p*-nitrophenol in resistant insects. (Solutions are listed in Section 34.2.1b.)

(i) Microtitreplate esterase assay

1. Homogenize mosquitoes in sodium phosphate-Triton (1 mosquito/mL).
2. In wells of the first and second columns, introduce 0.05 mL of sodium phosphate-Triton. In all other wells, introduce 0.05 mL of homogenate.
3. In wells of the first column, add 0.1 mL of each naphthol standard solutions. In all other wells, add 0.1 mL of substrate solution.
4. Incubate for 30 min at RT.
5. Add 0.1 mL of stain solution to all wells.
6. Read OD at A_{600} after 2 to 10 min. Specific esterase activity can be estimated from a standard curve constructed from naphthol standard solutions.

(ii) Visualizing esterases after electrophoresis

After an electrophoretic separation, the esterases of an extract can be revealed by incubating the gel for 10–30 min in 100 mL of 0.1 M sodium phosphate (pH 6.5) containing 10 mL of alpha/beta-naphthyl acetate solution. Upon addition of 300 mg of Fast Garnett GBC to the above solution, esterases preferentially using alpha-naph-

thyl acetate appear as blue spots whereas those preferentially using beta-naphthyl acetate appear as red spots. In *Culex* mosquitoes, the first esterases have been named esterases A, the second esterases B.

(c) Activity of MCE

MCE activity can be studied by taking advantage of ethanol formation upon the degradation of malathion. (Solutions are listed Section 34.2.1c.)

1. Homogenize insects in 0.1 M sodium phosphate pH 6.5 (w:v). Filter the homogenate through glass wool.
2. For spectrophotometry assay: 0.715 mL 0.3 M Tris-HCl pH 8.5, 0.2 mL homogenate, 0.025 mL ADH (30 U), 0.05 mL 40 mM NAD, 0.01 mL NAD-diaphorase (0.05 U), 1 mg INT, and 0.015 mL malathion (150 mM). Measure OD variations at A_{500} for 20 min. MCE activity toward malathion, which corresponds to the rate of INT reduction, is estimated using an extinction coefficient of 0.01375 $A_{500}/\mu M$.
3. For visualizing MCE after electrophoresis: Incubate gel 30 min in 0.1 M sodium phosphate pH 6.5, then 16 h in the following solution: 0.1 M sodium phosphate pH 6.5, 10 mM MgCl2, 0.04% w/v NAD, 0.01% w/v PMS, 0.01% w/v NBT, 0.02% w/v malathion and 300 U ADH. MCE enzymes appear as dark blue spots.

(d) Activity of GST

There are many available substrates to measure GST activities (Habig *et al.*, 1974. Among them two closely related substrates (DCNB and CDNB) have to be used, as GST-mediated resistance is often associated with a modification of the ratio of these activities. (Solutions are listed in Section 34.2.1d.)

1. Homogenize insects in 0.02 M sodium phosphate pH 6.9 (1/10, w/v). Centrifuge 10 min at 10 000 **g**.
2. Mix in the reaction cuvette: 0.01 mL DCNB or CDNB, 0.01 mL GSH solution, 0.89 mL 0.02 M sodium phosphate pH 6.9, 0.1 mL insect extract. In the blank, replace buffer for the insect extract. Measure the optical density increase at A_{340}. In order to calculate the number of nanomoles of substrate conjugated with GSH, it is possible to use the molar absorption coefficients calculated by Habig *et al.* (1974), i.e. EM (GSH/CDNB) = 9.6/mM/cm or EM (GSH/DCNB) = 8.5/mM/cm.

(e) Activity of MFO and cytochrome P450 content

MFO activities can be studied either by using chromogenic substrates or by measuring the amount of cytochromes P450 which are haemoproteins with characteristic optical spectra. Frequently eye pigments interfere with P450 measurement, and care must be taken to eliminate them. In addition, the thorax contains muscles with large quantities of mitochondria rich in cytochromes and must also be avoided. In consequence, it is preferred to work with abdomen extracts. (Solutions are listed in Section 34.2.1e.)

1. Preparation of microsomes (Note 1). Crush abdomens in PMSF-phosphate buffer (1/10; w/v). Centrifuge at 9000 **g** for 15 min. Filter the supernatant through cheese cloth (P450 content and MFO activities can be measured on this solution if there is no ultracentrifuge in the laboratory, but results are poor). Centrifuge the filtrate for 1 h at 150 000 **g** (4°C); resuspend the pellet in PMSF-

phosphate/glycerol (1/1 insect weight at the beginning/vol of buffer. This ratio has to be adapted with the P450 concentration in the extract).

2. Measure of P450 content: Fill two spectrophotometric cuvettes with the pellet suspension. Reduce the content of the two (control and assay) cuvette with dithionite (put directly in the solution a little amount of powder taken on a needle). Gently bubble CO in the assay cuvette. Leave to stand for 1 min. Monitor the spectrum from A_{500} to A_{400}; this monitoring is repeated until the spectrum is maximum. The amount of P450 (in nmole/mg microsomal proteins) is calculated by the following formula:

$$(OD_{490} - OD_{450}) / (91 \times \text{mg microsomal proteins}) \times 10^{-6}$$

3. MFO activity: MFO activities can be studied with many substrates. Activity on *p*-nitroanisole can be measured with a photometer, but this method is less sensitive than those using fluorogenic substrates (ethoxycoumarin or ethoxyresorufin) which require a fluorimeter. We describe here the measurement of 7-ethoxycoumarin dethylase which is one of the most commonly used. Standardize the fluorimeter with: 7-hydroxycoumarin solution (0.001 mL), ethyl acetate (0.75 mL), PMSF-phosphate/ethanol (0.75 mL). Prepare the assay mixture with: extract (*x* mL), ethoxycoumarin (0.005 mL), NADPH (0.0125 mL), PMSF-phosphate buffer (up to 0.5 mL). For the control (time 0) stop the reaction immediately by adding ethyl acetate (1.5 mL) to the assay mixture and stirring. For the assay, incubate at least 30 min before stopping the reaction with ethyl acetate (1.5 mL) and stirring. Fluorescence is measured on a Jobin Yvon fluorimeter (Gain 1, Energy 5) on the ethyl acetate extract (0.75 mL) to which is added PMSF-phosphate/ethanol (0.75 mL).

34.3.2 Purification of the proteins encoded by resistance genes

(a) Purification of AChE

Acetylcholinesterase may be purified by affinity chromatography using a ligand specific for the active site. Following this step, several additional methods may be used for further purifications, taking advantage of the glycolipid present at the C-terminal end of the protein (Triton X-114 partitioning), the glycosylation (concavalin-A affinity chromatography), the charge (DEAE chromatography), or molecular weight (membrane filtration) of the protein. (Materials are listed in Section 34.2.2a)

1. Extract preparation: Grind the insects in 20 mM Tris-HCl pH 7, 0.1% Triton X-100 (1:5 w/v). Filter the extract through a cheese cloth and centrifuge for 10 min at 10 000 g. Concentrate the supernatant by adding ammonium sulfate to 70% saturation (0.4 g/mL) and mix for 4 h at 4°C. Centrifuge for 30 min at 10 000 g, and dissolve the precipitate in 20 mM Tris-HCl pH 7, 0.1% Triton X-100. Dialyse 3× against the same buffer to eliminate the ammonium sulfate.
2. Affinity chromatography: Load the sample on the column. Wash with 20 mM Tris-HCl pH 9.5, 0.1% Triton X-100, and elute the bound cholinesterase with 20 mM Tris-HCl pH 9.5, 0.1% Chaps, 10 mM procainamide, 1 M NaCl.
3. Triton X-114 partitioning: Precondensed Triton X-114 is prepared by mixing Triton to 20 mM Tris-HCl pH 7 (6% w/v). Keep the solution at 5°C for 15 min

until the solution is perfectly clear. Promote phase separation at 37°C for 10 min, and centrifuge at 10 000 **g** for 10 min at RT. Discard the upper phase (aqueous phase) and add the detergent phase to the sample (1:1 v/v). Mix the two phases at 5°C and separate them at 30°C, as previously. Keep the detergent phase which contains the larger quantity of native cholinesterase (Note 2).

4. Concavalin-A sepharose chromatography (Note 3). Bind the sample in a buffer containing a detergent, 1 M NaCl and 1 mM $CaCl_2$. Elute the bound proteins in 20 mM Tris-HCl pH 7, 0.1% Triton X-100, 10% methyl-mannose.
5. DEAE chromatography: To purify by using the charge, load the sample onto a DEAE chromatography column in 20 mM Tris pH 9.5, 0.1% Triton X-100. Release of the protein is obtained by performing a ionic strength gradient by adding from 0–500 mM NaCl to the same buffer. Usually insect cholinesterases go through at around 200 mM NaCl.
6. Membrane filtration: To purify by using the size of the protein, charge the sample onto a membrane with a cut off at 50 kDa and dialyse. The cholinesterase is retained while smaller proteins are eliminated (100 kDa cut off is not enough to discriminate cholinesterases).

(b) Over-produced esterases

This method was used to purify over-produced esterases A and B of *Culex pipiens* (Fournier *et al.*, 1987). These esterases use naphthyl acetate as substrate and all enzymatic assays were done as described in Section 34.3.1b. Materials are listed in Section 34.2.2b.

1. Extract preparation. Homogenize insects in 0.025 M imidazole (pH 7.4). Filter the extract through glass wool. Centrifuge the filtrate for 15 min at 15 000 **g**; Keep the supernatant. Resuspend the pellet in 0.025 M imidazole (pH 7.4) and centrifuge as before. Mix the two supernatants.
2. Chromatofocusing. Determine the approximate pI of the esterases by isoelectric focusing in a 3–10 gradient pH using prepacked plates from Pharmacia and following their procedure (Note 4). Prepare the chromatofocusing column according to Pharmacia. Load the extract through the column equilibrated at pH 7.4. Wash the column with 0.025 M imidazole (pH 7.4) until no more protein is detected in the washing buffer. Prepare elution Pharmacia Polybuffer during the washing time. Elute the proteins in 4 mL fractions. Keep fractions with esterase activity.
3. Denaturing electrophoresis. The proteins of each fraction are then separated according to their molecular weight by SDS electrophoresis. To identify esterase(s) among the various proteins sharing the same pI, they are labelled with ^{14}C-DFP as follows. Mix enough ^{14}C-DFP (diluted in acetone) and esterase fraction until esterase activity is no longer detected. Precipitate proteins by mixing the solution with –20°C cold acetone (1 proteic solution: 8 of cold acetone, v/v). Centrifuge for 10 min at 5000 **g**. Resuspend proteins in Laemmli buffer so that the concentration of the proteic solution has increased 10-fold. Perform a denaturing electrophoresis with an aliquot of the suspension. Stain the gel with Coomassie Blue and perform autoradiography or fluorography to localize esterase(s) among the various proteins on the stained gel. Perform a second denaturing electrophoresis with the remaining labelled solution, stain the gel lightly, cut out the band(s) corresponding to the esterase(s) and wash extensively in 0.02 M sodium phosphate (pH 7.0).

(c) Purification of MCE

This method has been used by Haubruge (1995) to purify the carboxylesterase responsible for malathion resistance in *Tribolium castaneum*. Insect homogenates are carried out as in Section 34.3.1c. Materials are listed in Section 34.2.2c. All manipulations are done at 4°C.

1. Precipitation of MCE: Add ammonium sulfate to the insect homogenate (w/v) and mix thoroughly for 30 min. Centrifuge for 15 min at 20 000 **g**. Keep the supernatant and bring its concentration to 80% (w/v) of $(NH_4)_2SO_4$. Mix for 30 min. Centrifuge as before. Suspend the pelleted proteins in 10 mM Tris-HCl pH 8.0. Eliminate the excess $(NH_4)_2SO_4$ by chromatography through Sephadex G25. Pool all fractions containing MCE activity (= solution A).
2. Gel filtration chromatography. Load 5 mL of solution A on the Sephadex G200 gel. Elute with 10 mM Tris-HCl pH 8.0 with a flow of 0.075 mL per min. Measure MCE activity in each fraction and pool those containing activity (= solution B).
3. Ion exchange chromatography. Load solution B on the DEAE fractogel column. Wash the column with 10 mM Tris-HCl pH 8.0 until no more protein is detected. Elute by a linear gradient of NaCl done between 10 mM Tris-HCl pH 8.0 and 10 mM Tris-saline pH 8.0 under constant flow (0.8 mL/min) in fractions of 4 mL. Pool all fractions with MCE activity (= solution C).
4. Hydroxyapatite chromatography. Dialyse solution C against 10 mM sodium phosphate pH 6.8. Lyophilize and resuspend the sample in 10 mM sodium phosphate pH 6.8. Load sample on the hydroxyapatite gel and wash the column with 200 mL of 10 mM sodium phosphate pH 6.8. Elute proteins in a gradient from 10–400 mM sodium phosphate pH 6.8 under a constant flow (1 mL per min) in fractions of 3 mL. Pool fractions containing MCE activity (= solution D).
5. Isoelectric focusing. A preparative isoelectrofocusing, between pH 3.5 and 9.5, was performed using a specific material called Rotofor. Dialyse solution D against pure water and mix it with ampholines (750 mL) pH 3.5 and pH 9.5 from Biorad. Introduce this solution in the focusing chamber of the Rotofor with 0.01 M H_3PO_4 at the anode and 0.01 M NaOH at the cathode. Run the electrofocusing at 2000 V, 50 mA, 12 W for 4 h. Recover each electrofocused solution under vacuum in 20 fractions of 2.5 mL. Measure MCE activity in each fraction.
6. Nondenaturing polyacrylamide gel electrophoresis. This was done in order to increase the purity of the MCE after isoelectrofocusing. Dialyse the samples collected during the isoelectrofocusing with 10 mM sodium phosphate pH 6.8 for 16 h at 4°C, and then lyophilize them. Solubilize the powder in Tris-glycine. Load samples in a preparative electrophoresis apparatus (PrepCell - Biorad with gels of 10 cm in height, T = 8% and C= 3%), after filling the electrophoretic tanks with Tris-glycine and the elution chamber with Tris-saline. Run the electrophoresis with 20 W and 250 V until the tracer dye reaches the bottom of the gel, then increase the voltage to 360 V. An elution flow of 2.5 mL per min lasts at least 4 h.

(d) Purification of GST

GST can easily be purified using one of the several commercially available gels which fix these enzymes due to their ability to bind reduced glutathione (GSH). We describe here the method used to purify GST from *Culex pipiens*. All experiments were performed between 4 and 8°C. Materials are described in Section 34.2.2d. and methods to measure activity in 34.3.1d

1. Preparation of extracts. Grind adult mosquitoes in 0.02 M sodium phosphate pH 6.9 (1/10 w/v). Centrifuge the extract twice at 10 000 g for 10 min. Centrifuge the supernatant at 100 000 g for at least 1 h. Cytosolic soluble GSTs are located in the supernatant. The first extract and the supernatants (obtained above) are filtered through glass wool before proceeding to the next step.
2. Chromatography. Load the glutathione-agarose column with the supernatant obtained after the 100 000 g centrifugation (10 mL of supernatant were sufficient for our extracts). Wash the gel with 0.02 M sodium phosphate pH 6.9 until no protein can be detected in the washing solution. Elute the fixed proteins with Tris-GSH. Pool fractions containing GST activity (Note 5).

(e) Cytochrome P450 purification

These proteins have been purified from Diptera, mainly housefly (Scott and Lee, 1993) and *Drosophila* (Waters *et al.*, 1992). Researchers who want purified P450 from mosquitoes have to rely on the original publications.

34.3.3 Preparing antibodies from purified proteins

The solution containing the purified protein is mixed with the same volume of complete Freund adjuvant (Sigma) and injected intradermically into two rabbits. Boosters are done 15 days after the first injection, and then monthly. The injected solution is prepared as for the first injection, but the complete Freund adjuvant is replaced with incomplete Freunds.

Blood is sampled 2 months after the first injection, and then every 15 days. The reactivity and specificity of the sera are checked by western blotting.

34.3.4 Cloning resistance genes

(a) Cloning methods requiring protein purification

Once a purified protein has been obtained, there are several strategies to clone the corresponding gene.

If antibodies have been obtained, they can be used to screen a cDNA expression library. The techniques to construct and immunologically screen such a library are described in Chapter 19). In general, this strategy is used when the protein is quantitatively important, indicating that there is a good probability of obtaining a large quantity of the corresponding mRNA. To our knowledge, very few cDNAs corresponding to an insect resistance gene have been cloned according to this strategy. They concern the over-produced esterases from *Myzus persicae* (Field *et al.*, 1988) and from *Culex pipiens* (Mouchès *et al.*, 1986; Vaughan and Hemingway, 1995) in which esterases represent between 3 and 10% of the total soluble proteins, and a cytochrome P450 from housefly (Feyereisen *et al.*, 1989).

If enough pure protein has been obtained, peptides derived from the protein can be microsequenced, usually in a specialized laboratory. In general, the number and the length of the peptides that are sequenced is a compromise between the cost and obtaining a useful sequence, i.e. a sequence including several amino acids corresponding to codons with a low degree of degeneracy (the best amino acid is methionine encoded by a single codon, ATG).

Oligonucleotide sequences corresponding to the peptidic sequences are then obtained and used for screening either a cDNA library (Chapter 19) or a genomic library (Chapter 17), or for performing PCR (polymerase chain reaction) on genomic DNA, or RT-PCR (reversed transcriptase-polymerase chain reaction) on mRNA (Chapter 21). If the cDNA obtained is only partial, a full length cDNA can be acquired by RACE (Rapid Amplification of cDNA Ends) on the 5′ mRNA end of the gene. Although RACE can work with total RNA, better results are obtained with poly(A)$^+$ RNA which is obtained as follows (materials are listed in Section 34.2.3a; see also Chapter 15):

1. Grind insects in guanidinium buffer (1.5 mL) in a microfuge tube.
2. Add 0.5 mL of guanidinium buffer, and centrifuge for 20 min in a microcentrifuge. Transfer the supernatant to a 2.2 mL tube. Add acid phenol (0.5 mL) and chloroform (0.5 mL) and centrifuge for 20 min.
3. Transfer the aqueous layer to a tube (2.2 mL), add 0.25 mL ethyl ether, and centrifuge for 5 min. Remove ether, add 0.6 mL isopropanol, put the tube on ice (15 min), and centrifuge for 15 min. Remove isopropanol with a Pasteur pipette. Add distilled water to the pellet.
4. Separate Poly(A)$^+$ from the total RNA on polydT gels.
5. RACE is performed as described by Promega. It is necessary to perform several experiments in order to find the working temperature.

(b) Cloning methods based on nucleotide sequence homologies

Genes homologous to those involved in insecticide resistance have been isolated in noninsect organisms, mainly in vertebrates. These genes may be used as probes to screen libraries or to design PCR primers to amplify the genes of interest. To design PCR primers, it is assumed that the gene has been conserved during its evolution in various organisms, and regions of high homologies are identified using one of the many available computer softwares. The 'conserved sequences' are used to deduce the oligonucleotide sequences that will be used for PCR or RT-PCR on genomic DNA or mRNA as described above.

A second possibility is to first clone the homologous gene in *Drosophila*. Several specific techniques, based on genetics, are available in this species. First, the gene has to be mapped precisely on its chromosome. This step is performed by using already isolated mutations, and/or by creating new ones by mutagenesis. Then, the region of interest may be cloned by chromosome walking or transposon tagging. Transcription mapping and sequencing allow the gene to be characterized, and its role in resistance may be confirmed by P-element mediated transformation and by *in vitro* expression. The techniques used in *Drosophila* have been described in various laboratory books (Roberts, 1986; Ashburner, 1989). They have been used successfully to clone acetylcholinesterase (Hall and Spierer, 1986), the Na^+ voltage-dependent channel protein (Loughney *et al.*, 1989), the GABA receptor of the GABA-gated chloride channel (ffrench-Constant *et al.*, 1991) and some esterases (Newcomb *et al.*, 1995). If we add to this list several glutathione transferases and oxidases which have been cloned with other techniques in *Drosophila*, clones of most of the genes coding for proteins known to be involved in insecticide resistance are now available in this species. However, 'genetic cloning', because it allows genes to be cloned even if their product is not known, may be extremely valuable to clone the resistance genes involved in regulation.

34.3.5 Nature of the mutations conferring resistance

(a) Characterization of point mutations

Sequencing a resistance gene (Chapter 25) and comparing its sequence with the susceptible gene, may disclose one or several point mutations changing the primary amino acid sequence of the protein. Once such a mutation is discovered, it must first be verified that the mutation is not a cloning (or PCR amplification) artefact by sequencing another independent clone. To assign a particular mutation to resistance, a possibility is to mutagenize the susceptible gene so that it contains only this mutation and to express the susceptible and mutagenized genes *in vitro*.

Several protocols may be used for site-directed mutagenesis. It is possible to hybridize a mutated oligonucleotide to single-stranded DNA; the unmodified regions are polymerized and the wild type DNA used as template is eliminated. Another possibility is to use PCR techniques with one of the primers carrying the mutation of interest. The gene is sequenced to verify the presence of the wanted mutation and the absence of unwanted ones. Several protocols and kits to carry out *in vitro* mutagenesis are available; they can be found in Sambrook *et al.* (1989) and in the current molecular biology protocols.

E. coli may be used to express glutathione transferases. Several systems and protocols to produce proteins in *E. coli* are described in current molecular biology protocols. Glutathione transferases are expressed at a high level (up to 20 mg/L) and are active.

Baculovirus infected cells may be used to express acetylcholinesterases, esterases, oxidases and the GABA receptor. Protocols are described in O'Reilly *et al.* (1992). The expression system involves cloning the gene in a plasmid under the control of the polyedrin promoter in a 'transfer vector'. This vector has an *E. coli* origin of replication and an antibiotic resistance gene for propagation in *E. coli*, allowing construction work. Flanking sequences, originating from the viral DNA, allow recombination when the viral DNA and the transfer vector are cotransfected into *Spodoptera frugiperda* cells. When recombination between the two DNAs takes place, a new virus expressing the protein instead of polyedrin may be selected. To avoid the tedious selection of recombinant virus from the 'wild type', the DNA virus may be cut by a restriction enzyme between the two recombination sites, so that the linearized nonrecombinant virus does not grow and the circular recombinant virus expressing the protein may be quickly obtained (Kitts *et al.* 1990; a kit is available from Clontech).

Xenopus oocytes may be used to express the GABA receptors, Na^+ voltage-dependent channel protein and acetylcholinesterases. Two different strategies may be used: injection of cRNA in the cytoplasm, or injection of the gene under the control of an eukaryotic promoter in the nucleus. The second method gives larger amounts of protein but is not reproducible between oocytes. Protocols for nuclear injection are described in Bertrand *et al.* (1990).

(b) Characterizing quantitative mutations

Resistance due to over-production of an enzyme can be investigated at the protein level using specific antibodies, or at the RNA level using nucleic acid probes. Quantification of structural genes in genomic DNA will determine whether the over-production of protein and of mRNA is due to gene amplification.

(i) Protein quantification by dot–blot immunoassay
This technique was used to quantify esterase A and B proteins in OP resistant *Culex pipiens* mosquitoes (materials are listed in Section 34.2.3b).

1. Preparation of the blots: Grind insects in 25 mM imidazole pH 7.4 (0.01 mL/mosquito). Prepare serial dilutions of the homogenate [1:2, 1:4, 1:8 ...(v/v)], and transfer 0.005 mL of each homogenate solution onto a nitrocellulose filter. After having deposited 10 homogenates (or serial dilutions), immerse the filter in a solution containing 1% of SDS and 1% of β-mercaptoethanol for 10 min. Transfer filter to a solution containing 10% acetic acid and 25% isopropanol. Rinse the filter in H_2O, and allow to dry (Note 6).
2. Antibody reaction: Incubate filter in TS-SBA for 30 min. Transfer the filter in TS-SBA/antiserum for 30 min. Wash the filter (3× for 10 min in TS-Triton buffer and 2× for 10 min in TS buffer). Soak the filter in TS-SBA/AP for 30 min. Wash the filter. Dry the filter on tissue paper. Incubate the filter in AP stain. Stop reaction when colour sufficient (generally a few minutes) by transferring to Tris-EDTA pH 8. Allow to dry, read results and store.

(ii) Quantifying mRNA
Messenger RNA (mRNA) can be quantified by dot–blot according to the method described in Sambrook *et al.* (1989). Here we describe a method for quantifying mRNA on northern blots.

RNAs are fractionated using classical northern blotting (Chapter 20). The blotted RNAs are successively hybridized with a probe specific for the gene of interest and, after this probe has been stripped from the filter, with an actin SC probe. It is thus possible to obtain two autoradiographs of the same blot: one depicting the expression of the resistance gene, the other depicting the actin SC expression. As the latter is a 'house-keeping' gene, it provides an internal control of the amount of RNA loaded in each lane.

The two autoradiographs are scanned together (with a HPIICX scanner), and the densitometric image analysis is performed with, for example, NIH Image software running on a personal computer. NIH Image is available by anonymous FTP at 'zippy@nimh.nih.gov'. The arbitrary densitometric values obtained for the resistance 'RNA' are normalized using the corresponding lanes with the actin SC gene. Using this method we have been able to compare the expression level of a gene in various environmental conditions or in various strains.

(iii) Quantifying genomic DNA
A dot–blot method using serial dilutions of genomic DNA provides an easy way to quantify a gene amplification. The one-copy reference is given by a susceptible strain. Follow the protocol associated with the Amersham dot–blotter apparatus.

34.3.6 Detection of resistance genes in natural populations

(a) Test TEP for detecting AChE genotypes
This technique was developed for *Culex pipiens* mosquitoes, and allows the AChE genotypes (SS, RS and RR) of insects from France to be identified as described by Raymond *et al.* (1985). With other resistant AChE alleles, the correct concentration of

insecticide which inhibits AChE completely in SS individuals, but does not affect the AChE of RR genotypes, has to be determined. The test uses microtitreplates of 96 wells with a flat bottom. Three wells are needed for each mosquito, and it is recommended that three reference mosquitoes (SS, SR and RR genotypes) are run with each test. Thus a total of 29 insects with an unknown genotype can be run simultaneously (materials are listed in Section 34.2.3c).

1. Homogenate preparation. Homogenize each mosquito in a microfuge tube with 0.25 mL of SP buffer. Centrifuge for 5 min at 5000 **g** (in a microfuge) or allow to stand at RT for a few hours.
2. Assay: Mark on the microtitreplate, four columns of three wells, named (a), (b) and (c). Each well (a) and (c) of a row in each of the four columns receive 0.1 mL of the same mosquito homogenate, whereas row (b) receives 0.1 mL of water. Add in wells (c) of each row 0.01 mL of 0.001 M propoxur solution. Allow to stand for 15 min at RT. Add 0.1 mL of stain solution in all wells. Allow to stand at RT and read the test after 15 min and again after 1 h.
3. Interpretation of the test is based on the difference in colour intensity developed in the three wells corresponding to one mosquito, and three cases are possible. If the colour in well (b) and (c) has the same low intensity as that in well (a), the mosquito was a homozygote SS. If wells (a) and (c) display the same strong intensity while intensity in well (b) is low, the mosquito was a homozygote RR. If colour in well (c) is intermediate between that in wells (a) and (b), the mosquito was a heterozygote SR.

(b) Measuring MFO activities in single insects

Recently methods have been published for measuring MFO activities on a single *Drosophila* abdomen (Sousa *et al.*, 1995) or on single *Heliothis virescens* (Rose *et al.*, 1995). For *Drosophila*, it is possible to use either microsome extracts or whole abdomen. Experiments are performed in 96 wells microtitreplates. Solutions are described in Section 34.2.1e.

1. With microsomal fractions (prepared as indicated in Section 34.3.1e). Prepare assay mixture in each well: extract (× mL), 7-ethoxycoumarin 0.4 mM, 0.001 M NADPH, PMSF-phosphate buffer up to 0.1 mL. Incubate 4 h at 30°C, then stop reaction by adding PMSF-phosphate/ethanol (0.1 mL). Fluorescence is measured directly on the microtitration plate with a Fluorolite 1000 (Dynatech, Guyancourt, France). Using excitation filter 390 nm, emission filter 450 nm, and an energy of 5 V.
2. With intact tissue. Put one abdomen in each well containing the solutions described above but omitting NADPH. Proceed as described above, but centrifuge the microtitre plates at 2000 rpm for 30 s in a Jouan N6 type centrifuge before incubation and when the reaction is stopped.

(c) Over-produced esterases

These can be detected in single insects by different techniques, including the microtitreplate assay or after electrophoretic separation as described in Section 34.3.1b, or by the dot–blot immunoassay as described Section 34.3.5b, providing that homogenates are done with single insects (adults or 4th-instar larvae).

(d) Identification of amplified esterase A or B haplotypes in C. pipiens

1. DNA extraction from single mosquitoes (Section 34.2.3d for materials). Homogenize gently and thoroughly each insect in 0.05 mL of extraction buffer A with a polypropylene pestle in a microfuge tube. Rinse the pestle with 0.1 mL buffer A in the tube. Add 0.1 mL of buffer B; mix thoroughly with a micropipette; leave on ice for 1 h. Spin at high speed in a microcentrifuge for 10 min; transfer the supernatant into a new microfuge tube (discard the pellet). Add 2 vol of ethanol, mix gently and leave at –20°C for 1 h or overnight. Spin at high speed in a microfuge for 10 min. Pour off the supernatant carefully. Add 0.5 mL 70% ethanol, allow to stand for 5 min, pour off the supernatant. Dry the pellet under vacuum for 10 min. Keep at –20°C until further processed.
2. DNA digestion. Before digestion: Add 0.017 mL of 10 mM Tris-HCI, 0.1 mM EDTA, pH8. Allow to stand for at least 1 h at 4–6°C. Add 2 μL of an appropriate restriction enzyme buffer, and 1 μL of restriction enzyme (e.g. *Eco*RI to detect the 9 kb fragment characteristic of the B2 haplotype). Digest DNA from reference strains with the same restriction enzyme.
3. Gel migration and transfer. Choose the appropriate agarose gel concentration (0.8% in general) and follow standard protocols for gel electrophoresis, DNA denaturation and transfer to a membrane (Chapter 20).
4. Hybridization. Label an A or B esterase probe with ^{32}P. Follow standard protocols (Chapter 20).

(e) Identifying point mutations in resistance genes

PASA (PCR amplification of specific alleles) is a promising technique for identifying specific mutations of resistance genes. It has recently been used to characterize a particular point mutation of the GABA receptor known to be responsible for dieldrin resistance in *Drosophila melanogaster* and various other insect species, including *Aedes aegypti* (ffrench-Constant *et al.*, 1994).

The principle of the technique is that, by placing the allele specific mutation at the 3′ end of the oligonucleotide, poor amplification of the other alleles, but specific amplification of the matching allele, can be achieved due to mismatch between the DNA template and the primer. ffrench-Constant and his collaborators have shown that in most cases either the forward or the reverse primer gives stronger discrimination between susceptible DNA and specific resistant alleles, and that superior discrimination depends on the concentration of $MgCl_2$ in the reaction. Furthermore, in order to guard against scoring false negatives, addition of a second set of allele-independent primers are included in the PCR. As for the PASA primers, the product of these primers is dependent upon the $MgCl_2$ concentration.

For *Aedes aegypti*, ffrench-Constant *et al.* (1994) obtained the best results with the mutation on the forward PASA primer, and specific amplification of the resistant allele alone was achieved at 2 mM $MgCl_2$ or above. PCR was conducted by adding 100 ng of genomic DNA to a 0.05 mL reaction containing 0.25 μM of the allele-specific primer and the allele-independent primer on the same strand, and 0.25 μM of the allele-independent primer on the opposite strand, 0.02 mM dNTPs and 1.5 U of *Taq* polymerase. PCR was carried out for 30 cycles with 1 min denaturation at 94°C, 1 min annealing at 53°C, 2 min extension at 72°C and a final extension at 72°C. PCR products are then electrophoresed in a 4% agarose gel and visualized with ethidium bromide. The primers were: 5′-CTACACCAGCACGTGTTT-3′ for the forward allele-

specific primer, 5′-GTACATCATTCTGGATCT-3′ for the forward allele-independent primer, and 5′-TACCATAAACGGTCAGAC-3′ for the reverse allele-independent primer. Defining primers for other vector species will require the GABA receptor gene to be isolated and sequenced.

NOTES

1. All manipulations are performed at 4°C or in ice.
2. Some hydrophilic cholinesterase may be also present in the aqueous phase due to previous degradation of the membrane form. Subsequent activity measurements or purification steps should be performed by diluting the detergent phase in a buffer containing 1% Triton X-100 to avoid clouding of Triton X-114.
3. It may be used to purify insect cholinesterases but the enzymes are often difficult to release from the gel, i.e. only 30–40% recovery.
4. In the case of *C. pipiens*, it was found that the pI of esterases A and B lay between 5 and 6, and chromatofocusing was performed in the pH range of 4 to 7.
5. By this method, two families of GST were purified. The members of each family can be further separated by ion exchange chromatography, or by chromatofocusing using NaCl gradient to elute the linked GSTs.
6. Filters can be stored in sealed plastic bags until further processed, several weeks if necessary.

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Part Six
Transformation Techniques and Viral Systems

CHAPTER THIRTY FIVE

Microinjection of mosquito embryos

Alison C. Morris

35.1 INTRODUCTION

Due to the recent interest in the development of novel genetic methods to control insect vectors of disease, a great deal of research has focused on the genetic manipulation of the insect vector (James, 1992). In the design of a control programme involving genetic modification of the insect vector, factors to be considered include the isolation of potentially useful target genes, appropriate tissue-specific control elements to direct their expression, and a means of introducing exogenous DNA into the insect genome.

Recent research has focused on the study of exogenous transposable elements, site-specific recombination systems (Morris *et al.*, 1991) and homologous recombination strategies as effective means to introduce DNA sequences into the genome. To facilitate germline transformation, the exogenous DNA must be introduced into the developing embryo prior to pole cell formation. To this end, techniques for microinjection of the mosquito embryo have been developed. To date, germline transformation experiments involving microinjection have been carried out with aedine mosquitoes (McGrane *et al.*, 1988, Morris *et al.*, 1989) and with *Anopheles gambiae* (Miller *et al.*, 1987). In addition, microinjection has proved to be an effective means of delivering recombinant DNA into *Aedes aegypti* embryos for analysis of embryo-specific putative promoter sequences (Morris *et al.*, 1995). Microinjection techniques used in these experiments will be described, and should be applicable to other species of mosquito.

The technique for microinjection of the mosquito embryo was based on that used to introduce DNA into the developing embryo of *Drosophila melanogaster* (Karess, 1985). At oviposition, the *Drosophila* embryo is bounded by two envelopes, an inner vitelline membrane and an outer opaque, white chorion. The chorion is rigid and can be removed by lightly stroking the embryo on double-sided sticky tape. Following slight desiccation, the embryos, bounded only by the transparent vitelline membrane, can then be injected using a drawn microcapillary needle connected by air-filled tubing to a 10 mL syringe. The optimum site of delivery of the DNA solution is the region of the yolk adjacent to the periplasmic space, a region of clear cytoplasm at the posterior pole, since it is in this region of the yolk that pole cell formation occurs, followed by their budding off into the periplasmic space. In *Drosophila*, this target region for injection is clearly visible through the transparent vitelline membrane. In contrast to

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Drosophila, the rigid, opaque chorion of the mosquito cannot be removed (Clements, 1992). Fundamental differences in the morphology of *Drosophila* and mosquito embryos led to the development of a modified technique for microinjection.

The mature embryos of aedine mosquitoes are black in colour and covered by transparent tubercles. The dorsal surface is convex with a flatter ventral surface. Oviposition is preferentially onto a moist surface rather than directly onto water, with the flatter ventral surface uppermost. The anterior end of the embryo is slightly wider than the posterior end and can be distinguished by the presence of the micropyle, a funnel-shaped membrane with its open end protruding slightly beyond the surface of the embryo. This serves as the point of sperm entry during fertilization.

Anopheline embryos are boat-shaped with a more blunt anterior end, and are laid individually on the surface of water. The exochorion forms an air-filled sac on each side of the embryo, which causes them to lie with the convex, lower surface submerged in the water.

Mosquito embryos are bounded by a translucent exochorion. This fragile exochorion covers the more rigid endochorion. The endochorion may be 4–5 μm thick and gives the embryo its shape and rigidity. It is the principal mechanical protection for the egg while permitting gas exchange and minimizing water loss. The exochorion is very easily damaged, particularly in the manipulation of the embryo for microinjection. Removal of the exochorion reveals the smooth, shiny endochorion. In freshly oviposited embryos the endochorion is relatively soft and is a translucent white colour, but darkens to a pale grey colour approximately 1 h after oviposition and to a dark grey/black colour over a period of 2 h. Complete darkening to black takes 4–5 h, during which time it becomes progressively more rigid and brittle. The opaque endochorion cannot be removed from the embryo since no vitelline membrane is present at oviposition and the serosal cuticle, which lies beneath the endochorion, is not formed until 20 h (Clements, 1992).

After oviposition, the embryo may increase in size by up to 25% due to uptake of water over a period of 24 h. When the embryo has swollen to its maximum size and the endochorion fully darkened after 24 h and up to 72 h, it is still sensitive to desiccation. The period up to 72 h after oviposition is known as the conditioning period, during which time larval development occurs. Following complete embryonic development of aedine mosquitoes at 72–96 h, the embryos become resistant to prolonged desiccation of up to 4 months, though increasing the storage of the dried embryos beyond this may lead to a greater mortality. In contrast, completion of anopheline embryonic development must be followed by hatching since the embryos remain sensitive to desiccation.

35.2 MATERIALS

1. Double-sided sticky tape (Scotch™ or Sellotape™).
2. Halocarbon oil: Series 700 from Halocarbon Products, River Edge, NJ, USA. Water saturate by mixing with distilled water.
3. Microcapillaries: preferably with inner filament, outer diameter 1 mm, inner diameter 0.8 mm available from World Precision Instruments, Inc., Sarasota, FL, USA or Clark Electromedical Instruments, Reading, UK.
4. Electric needle puller: e.g. Flaming Brown Micropipette puller, Model P-87, Sutter Instrument Co., San Rafael, CA, USA.

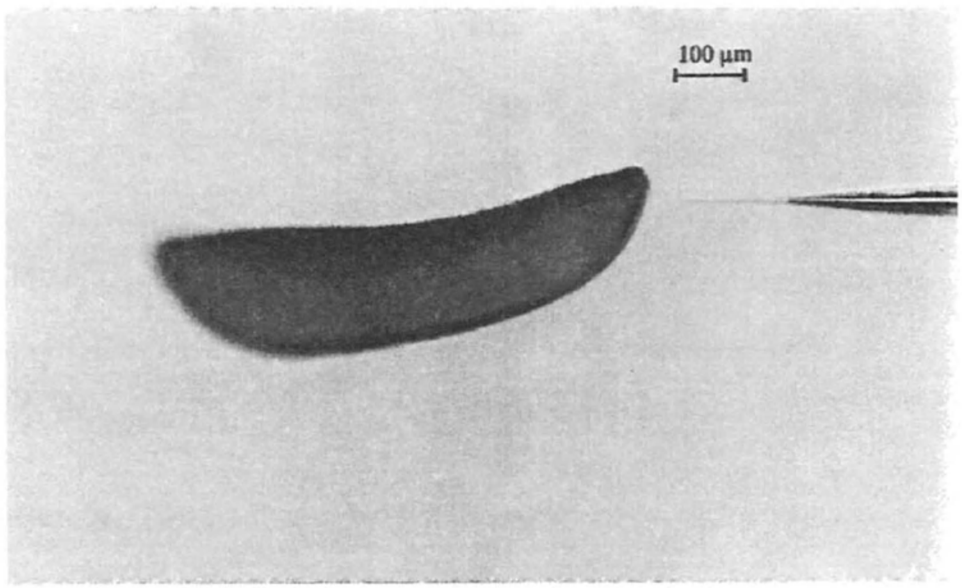
5. Plasmid DNA solution for injection should be applied to a CsCl gradient for purification (Sambrook *et al.*, 1989). Recovered plasmid DNA should be precipitated in absolute ethanol and 1/10 volume of 3 M sodium acetate pH 5.2. Pellet the DNA and wash with 70% ethanol before resuspending in injection buffer (5 mm KCl, 0.1 mM sodium phosphate pH 6.8) up to 1 mg/mL (Note 1).
6. Micromanipulator: e.g. Narishige MMN-333, US Narishige International Inc., New York, USA or Narishige International Ltd, London, UK.

35.3 METHOD

1. Transfer individual female aedine mosquitoes, 3–4 days post-blood meal, into glass or plastic tubes (3 × 8 cm) containing a plug of moist cotton wool at one end, covered by a circle of moist filter paper which serves as an oviposition site. A similar plastic tube containing 2 mL of water, lined with a 3 cm strip of filter paper at the bottom, will serve as an oviposition site for anopheline mosquitoes. The eggs will be laid on the surface of the water and will adhere to the filter paper on removal (Note 2). Mid-dark grey embryos approximately 90–120 min old are at the optimum age for injection.
2. Remove the oviposition paper from the tube and, using a dissecting microscope and fine forceps, orientate 20–30 embryos on the filter paper with their posterior poles aligned (Note 3).
3. Dry the oviposition paper thoroughly by pressing dry filter paper onto its edge. Attach a strip of double-sided sticky tape, approximately 3 × 10 mm to the edge of a plastic coverslip. Invert the coverslip and pick up the embryos from the oviposition paper so that their posterior poles are close to the edge of the coverslip. Insufficient drying of the oviposition paper will result in difficulty in transferring the embryos to the sticky tape.
4. Under the dissecting microscope (10× magnification), monitor desiccation of the embryos (Note 4). Adequate desiccation is dependent on the ambient humidity and is achieved when the upper surface of the embryo begins to bow slightly inwards. This may take approximately 15–60 s at RT, or may require 1–1.5 min in a desiccator.
5. Cover desiccated embryos with water-saturated halocarbon oil to prevent further desiccation and store in Petri-dishes lined with moist filter paper.
6. Injection needles are prepared by drawing capillary tubing into a fine tip of 3–8 μm with a shaft of approximately 100–300 μm using a micropipette puller (Note 5). Solutions for microinjection are subjected to a brief (5 sec) spin at full speed in a benchtop microcentrifuge to pellet debris. Injection needles are loaded by filling a hand drawn capillary tube (with a bore of approximately 0.5 mm) and using this to introduce fluid into the open end of the needle.
7. Connect the needle to an air-filled 20 mL syringe using air-filled plastic tubing (Note 6).
8. Microinjections are performed within 2 h of oviposition using a micromanipulator (Note 7), at 100× magnification. Aedine embryos should be injected horizontally to prevent tearing of the endochorion. If necessary, a raised microscope stage may be prepared by stacking 4–5 microscope slides and fastening with double-sided sticky tape. Place the coverslip carrying the embryos onto the raised stage. Anopheline embryos are injected at a 15° angle and penetration

should be at the posterior pole, just in front of the posterior tip (Figure 35.1). An overhead light source will facilitate alignment of the injection needle with the appropriate plane of the embryo for injection.

A



B

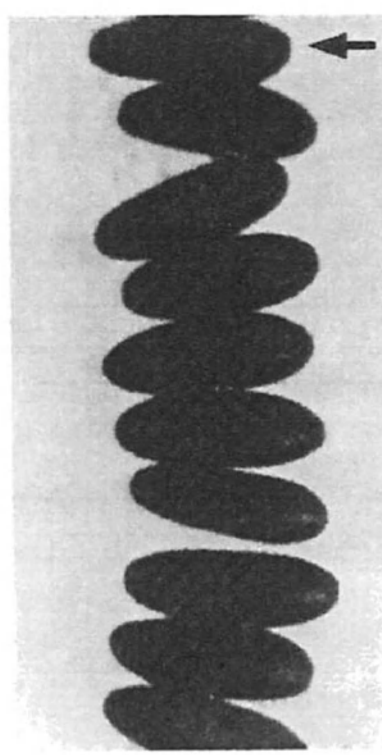


Figure 35.1 (A) Microinjection of an *Anopheles gambiae* embryo. The embryo should be microinjected just in front of the posterior tip as shown. (B) *Aedes aegypti* embryos aligned for microinjection. The posterior end is on the left. The arrow indicates an over-desiccated embryo as determined by the multiple indentations on its surface. Adequate desiccation results in a slight bowing of the surface or a single indentation.

9. Inject 0.2–0.5 nL of solution, corresponding to approximately 1–5% of the embryo volume. Control the injection volume by adjusting the injection pressure and time. The slightly bowed, desiccated embryos should regain their turgid appearance following injection.
10. Return the coverslip to the Petri-dish and return to insectary conditions for 20–30 min.
11. Pick embryos from the cover-slip using fine-forceps, place onto moist filter paper and return to insectary conditions for 3–4 days to allow for larval development. Anopheline embryos are placed onto moist filter paper lining a Petri-dish with water in the bottom. Emerging larvae will then swim into this water. Aedine embryos are stimulated to hatch by placing the filter paper in organically charged water, e.g. a solution of bovine liver powder or a grass/hay infusion (Note 8).

NOTES

1. DNA solutions at concentrations greater than 1 mg/mL are viscous and tend to block the injection needles. More dilute solutions can be used and may lead to increased survival of injected embryos, though decreasing the number of plasmid copies introduced may affect transformation frequencies or the level of reporter gene expression.
2. Larger oviposition pots may be placed into cages to collect embryos *en masse*. However, isolating single females results in synchronized batches of eggs of similar age and development. Injecting embryos <90 min old results in decreased survival rates. For transformation attempts using plasmid DNA, the embryos should be injected prior to pole cell formation which occurs at 2.5–3 h after oviposition. The endochorion of embryos >2 h old is too rigid to inject.
3. The air-filled sacs on the sides of the anopheline embryos make manipulation and alignment difficult. The exochorion may be removed by gently stroking the embryos on the filter paper or by treating for 3–4 min with a 0.25% sodium hypochlorite solution, prepared by diluting household bleach. A subsequent 5 min rehydration period in distilled water is necessary for embryo survival.
4. The exochorion is often detached or damaged by the manipulation of the embryos so that only the endochorion is visible. In embryos where the exochorion is still present, at least in part, the first sign of desiccation is the shrinking of the endochorion away from the exochorion giving the latter a silvery, translucent appearance. In embryos without the exochorion, desiccation is first obvious when the endochorion loses its shiny appearance. Further desiccation results in one or both of the upper sides of the embryo collapsing, so that the egg is eventually tri-radiate in section and, ultimately, flattened to a saucer shape. Several embryos may become over-desiccated before the majority of embryos reach a suitable stage of desiccation. These embryos must be discarded to avoid confusion over viability of injected embryos. Insufficient desiccation leads to leakage of cytoplasm on microinjection. If the embryos are allowed to collapse from over-desiccation at any time prior to completion of the conditioning period, they can be swollen to their original size by placing in water, though the embryo contents will be disorganized and the embryo inviable. This phenomenon could lead to some confusion during the process of microinjection in which the embryos are desiccated slightly as for *Drosophila* embryos. Over-desiccated

embryos may assume their original shape and appear viable following microinjection, when in fact their failure to hatch is due to procedures prior to the introduction of a DNA solution.

5. In the design of the microinjection needle, the short region of taper reduces breakage of the needle when puncturing the embryo and the very small tip diameter prevents tearing of the brittle endochorion. A tip of 1–2 μm is optimum for puncturing the endochorion, but tips of this diameter are very easily blocked by the contents of the embryo and by remnants of the exochorion on the outside of the embryo. For injection of anopheline embryos the tip of the needle must be formed into a sharp acrosome or bevel. This can be achieved by bringing one side of the tip into contact with a hot glass ball on a de Fonbrune-style microforge and immediately pulling away (Miller *et al.*, 1987). Alternatively, the needle tip may be bevelled using a K.T. Brown-type micropipette beveller (Sutter Instrument Co. Ltd.).
6. An automatic injection system (e.g. Eppendorf microinjector 5242) may be used, involving two positive pressure levels, injection and holding. This reduces blockage of the needle which may result from introducing the tip into an insufficiently desiccated embryo. An additional advantage of this system is that a reproducible amount of liquid may be delivered. However, a much simpler alternative is to apply pressure using an air-filled 20 mL syringe which is only slightly less efficient than the elaborate and more costly automatic systems.
7. Microinjections may be performed using a micromanipulator, though this need not be elaborate. The primary function of the manipulator is in holding the injection needle and bringing it into the correct plane of focus. Injection may be carried out by moving the needle or by moving the microscope stage.
8. A hatch rate of up to 50% may be achieved using this technique, though a rate of 15–25% is more routinely achieved.

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CHAPTER THIRTY SIX

Transfection of cultured mosquito cells

Ann Marie Fallon

36.1 INTRODUCTION

The technology for introducing chimeric DNA constructs into cultured cells provides an important new approach for analysing diverse aspects of gene expression. In particular, transfection studies with cell lines from mosquitoes are beginning to provide insights into the structural and functional features of genes, and their regulatory elements that play essential roles in the physiological processes that maintain disease transmission cycles. Functional assays with transfected mosquito cells will further enable the investigator to develop novel approaches, such as antisense nucleic acid technologies and suicide selection systems, whose implementation has potential applications for molecular disruption of disease transmission in transgenic mosquitoes.

36.1.1 Why cultured cells?

Among insects, gene transfer mediated by the transposable element P in the fruitfly, *Drosophila melanogaster*, serves as the current standard for comparable applications with other species. The focus of this section on cultured mosquito cells, rather than the intact organism, reflects the difficulties that have been encountered in efforts to extend to mosquitoes the P element-based techniques that have been so successful with *D. melanogaster*. It now appears quite certain that the P element from *D. melanogaster* does not function efficiently in mosquito embryos or cell lines, at least under conditions analogous to those used with *Drosophila*. Not only do few transformation events occur, but those that have been characterized result from illegitimate recombination, rather than transposon-mediated events (Miller *et al.*, 1987; McGrane *et al.*, 1988; Morris *et al.*, 1989).

As an alternative to P, native transposable elements from mosquitoes may eventually provide an embryo-based transformation technology. Several transposable elements, primarily from members of the genus *Anopheles*, have been identified (Besansky *et al.*, 1994), but extensive molecular characterization of their properties remains to be undertaken, and a mosquito element with the versatility of the *Drosophila* transposable element P has not yet been described. Similarly, heterologous transposition systems, such as the FLP-FRT site-specific recombinase system from yeast, have been of limited utility in mosquito embryos (Morris *et al.*, 1991). Finally, transfection of dissected tissue, such as mosquito salivary gland, using con-

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structs encoding highly expressed reporter genes provides a promising approach for identification of functional promoters, but quantitative interpretation of results from these experiments remains technically difficult, and for efficient transfection, the tissue must be treated with elastase to remove the basement membrane (Morris *et al.*, 1995). Such applications are limited by the small amounts of tissue available from dissected mosquitoes, and by the viability of these tissues *in vitro*. In contrast, gene transfer in cultured cells occurs with high frequency and reproducibility, facilitating studies that cannot yet be done in the organism itself.

36.1.2 Requirements for transfection of cells

To evaluate expression of purified DNA in cultured cells, two basic requirements must be satisfied. First, the transfected DNA construct must contain regulatory elements compatible with the transcriptional and translational machinery of the cell. Secondly, a physical or chemical means of successfully introducing purified DNA into the cells of interest must be identified. Established methods for introduction of purified DNA into cultured cells, including co-precipitation of DNA with calcium phosphate, use of various polycations such as DEAE dextran, polylysine, polybrene (hexadimethrine bromide), or commercial reagents such as lipofectin, and physical methods such as microinjection or electroporation, were generally developed with vertebrate cells and have only recently been extended to insect cells (Walker, 1989). For a given cell line, it remains difficult, if not impossible, to predict which protocol(s) will prove satisfactory, and conditions for effective transfection are generally identified by systematic trial and error.

(a) DNA constructs

DNA constructs for expression in transfected cells have two essential elements: a promoter, which directs transcription of the gene, and a reporter gene, usually encoding an easily assayed enzyme. Typically, the promoter and reporter genes derive from different sources, are chosen according to the specific needs of the investigator, and are constructed using standard recombinant DNA technology.

Promoters from mammalian genes, and even promoters from *D. melanogaster* genes, do not necessarily function well in mosquito cells. By virtue of the evolutionary conservation of many aspects of the heat shock response, however, the recombinant plasmid, hsp-cat 1, developed for *Drosophila* cells by Di Nocera and Dawid (1983) provided an important tool in our early efforts to optimize transfection procedures for *Aedes albopictus* mosquito cells. In hsp-cat 1, the reporter gene is the chloramphenicol acetyltransferase (CAT) gene from *Escherichia coli*, and CAT expression is regulated by a promoter from the *D. melanogaster* heat shock protein 70 (hsp70) gene. Strong and inducible expression of hsp-cat 1 in transfected C7-10 *Ae. albopictus* mosquito cells provided a sensitive assay for optimizing chemical methods to introduce purified DNA into these cells (Durbin and Fallon, 1985). Despite the advances that have been made over the past 10 years, hsp-cat 1 remains the construct of choice for adapting transfection procedures to other mosquito cell lines. We note here that the sister construct, *copia*-cat, which uses the promoter from the *D. melanogaster* transposable element *copia* (Di Nocera and Dawid, 1983), was poorly expressed in C7-10 mosquito cells (Fallon, 1989).

The specific method by which expression of a transfected gene is evaluated will depend upon the purposes of the individual investigator, the nature of DNA constructs available, and the properties of the reporter gene. Activity of many reporter genes can be detected by more than one isotopic, histochemical, or biochemical assay. For studies requiring some knowledge of transfection frequency, the investigator will find it advantageous to use a reporter gene that can be detected by a histochemical assay that allows transfected cells to be identified and counted, and by a biochemical assay to standardize total amounts of reporter gene activity per cell number or protein content. Here we describe the biochemical and histochemical assays used for detection of CAT enzyme in this laboratory.

(b) Chemical mediators for transfection

Because a systematic comparison of transfection protocols with different mosquito cell lines remains to be undertaken, we focus the present discussion on use of the polycation polybrene for both transient and stable transfection of *Ae. albopictus* cells. In this laboratory, use of polybrene as a chemical facilitator has been simple, reliable, and uniformly reproducible among various personnel. Polybrene is inexpensive and easily prepared, and causes minimal damage to treated cells. Moreover, the availability of selectable marker genes for mosquito cells (Shotkoski and Fallon, 1993) now offsets the somewhat low (1/10 000 cells) transfection frequency achieved with this reagent.

In contrast, our early attempts to transfect mosquito cells using DNA co-precipitated with calcium phosphate provided only marginal success (Fallon, 1984), and we have since learned that DNA–calcium phosphate co-precipitates can be quite damaging to C7–10 mosquito cells. Considerable experience in cell culture and attention to procedural details can, however, provide transfection frequencies ranging up to 1 per 1000 cells using calcium phosphate (Kjer and Fallon, 1991). Moreover, when cells are transfected using calcium phosphate, input DNA that remains unincorporated can be removed, facilitating analysis of gene copy number and expression using standard hybridization procedures. For the interested reader, a comprehensive summary of our experience with calcium phosphate-based procedures may be found in the reference by Kjer and Fallon (1991), and a rare side-by-side comparison of various transfection techniques with insect cells from the cotton boll weevil, *Anthonomus grandis*, is provided by Stiles *et al.* (1992).

(c) Transient versus stable transfection

Transfection protocols are typically described in terms of transient (hours to days) or stable (weeks to months), depending on when expression of the introduced DNA is assayed, relative to the time of transfection. Transient expression of foreign genes is technically simpler, but is limited by promoter strength and by the proportion of cells that incorporate foreign DNA (the transfection frequency). It is often generalized that transiently-transfected cells maintain foreign DNA extrachromosomally, and that this DNA is gradually lost during subsequent cell divisions. In contrast, after stable transfection accompanied by selection for expression of a marker gene, such as the mosquito dihydrofolate reductase gene (Shotkoski and Fallon, 1993) or the hygromycin phosphotransferase gene (Monroe *et al.*, 1992), the DNA is more likely to have become integrated into chromosomal material. Although these assumptions have provided reasonable paradigms for our work with *Ae. albopictus* cells, to our knowledge they have not been tested rigorously with any insect cell culture system.

36.1.3 Mosquito cell lines

As the use of transfection technologies becomes more widespread, and work with mosquitoes expands to include diverse vector–pathogen interactions, new investigators will wish to consider carefully which cell line(s) and culture media best suit their projected needs. Mosquito cell lines have been developed from members of the genera *Aedes*, *Anopheles*, *Culex* and *Toxorhynchites*, and tissues of origin include embryonic cells, larval cells, and adult ovaries (Kurtti and Munderloh, 1994). As is the case for insect cell lines in general, the precise identity of cells from which a given continuous line derives is typically unknown. New approaches to insect cell typing using monoclonal antibodies (Willott *et al.*, 1994) should eventually provide useful information as to which cells proliferate *in vitro*, giving rise to continuous lines, and to what extent these lines retain the differentiated properties of their tissues of origin.

Properties of a cultured cell line that may be of particular interest to the new investigator include plating efficiency and growth rate, requirements for serum and/or other supplements, ability to grow in monolayer and/or suspension culture, freedom from endogenous viruses and mycoplasma, and their capacity for other experimental manipulation, such as fusion, cell cycle synchronization, or radiolabelling with a particular precursor. In addition, a diagnostic karyotype, protein profile, or other biochemical marker by which the identity of each line can be periodically confirmed is of potential value, particularly in laboratories where multiple lines are maintained. Finally, it is also useful to establish criteria by which cell lines can be confirmed as to the mosquito species from which they originated. For cells from *Ae. albopictus* and *Ae. aegypti*, we have found that the electrophoretic mobility of the small heat shock proteins is diagnostic for species verification (Lan and Fallon, 1990) (a brief comparison of two commonly-used *Ae. albopictus* cell lines is provided in Note 1).

Research in the author's laboratory has focused on *Ae. albopictus* cells derived from the ATC-15 line of Singh (1967), which was established from minced neonate larvae. Our initial interest in defining transfection conditions for C7-10 cells, which serve as a current standard, or wild type, clone (reviewed in Fallon and Stollar, 1987) was a natural extension of the earlier efforts of Victor Stollar and coworkers at the University of Medicine and Dentistry of New Jersey, who systematically applied the techniques and approaches of somatic cell genetics to studies on virus-induced cytopathology in these mosquito cells.

To summarize briefly, *Ae. albopictus* cells have been adapted to vertebrate tissue culture media and cloned (Sarver and Stollar, 1977; Igarashi, 1978; Mento and Stollar, 1978). In addition, protocols for mutagenesis and selection (Mento and Stollar, 1978), somatic cell fusion (Tatem and Stollar, 1986), and analysis of virus-induced cytopathology *in vivo* (Sarver and Stollar, 1977; Tatem and Stollar, 1986) and *in vitro* (Gillies and Stollar, 1982) are available. These technical achievements were facilitated by the gradual accumulation of genetically-marked lines, including two thymidine kinase deficient (TK^-) lines (Mento and Stollar, 1978; Mazzacano and Fallon, 1992) that provided potential recipients (Fallon, 1984; Mazzacano and Fallon, 1995) for transfection of a thymidine kinase (*tk*) gene that had been cloned from the Herpes Simplex virus (HSV) and successfully expressed in mouse Ltk^- cells (Wigler *et al.*, 1977). Because transfection studies are often facilitated by the use of appropriate somatic cell mutants (Note 8), the application of genetic techniques to other mosquito cell lines will prove to be an increasingly important consideration.

36.2 MATERIALS

36.2.1 Cell culture facility

1. Sterile tissue culture dishes, flasks, pipettes.
2. Haemocytometer or Coulter electronic cell counter (Coulter Corporation, Hileah, Florida).
3. Cell culture incubators adjustable to appropriate temperature and gas composition (Notes 2 and 3).
4. Laminar flow hood or equivalent.
5. Inverted microscope; adaptations for use of a camera and accessories.
6. Centrifuge (800–10 000 **g**).
7. Autoclave or oven for sterilization.
8. Liquid nitrogen cell storage facility (Note 1).

36.2.2 Transfection – general considerations

1. Mosquito cell line(s) (Note 1).
2. Culture media, serum (Note 2); serum-free medium.
3. Polybrene (hexadimethrine bromide, Sigma Chemical Co., St. Louis, MO): 10 mg/mL in distilled water; sterilize by filtration; store at 4°C; do not autoclave or freeze.
4. Purified DNA construct(s) in 10 mM Tris-HCl, 1 mM EDTA, pH 7.5; sterilize by gentle mixing with 0.1 vol of chloroform (Note 4).
5. Methotrexate [(+) amethopterin, Sigma]: Prepare a 1.0 mM stock solution by dissolving methotrexate in 1 mM NaOH; sterilize by filtration; adjust concentration based on the molar extinction coefficient of 23 000 at A_{257} in 0.1 M NaOH (Fallon, 1984).

36.2.3 Assays for gene expression

(a) Biochemical assay for CAT

1. Phosphate-buffered saline (PBS).
2. Sonicator.
2. 0.25 M Tris-HCl, pH 7.5.
3. ^{14}C-chloramphenicol (45 Ci/mM, New England Nuclear, Boston, MA).
4. Acetyl coenzyme A, sodium salt (to be distinguished from Coenzyme A; Sigma); prepare a 4 mM solution in 0.25 M Tris-HCl, pH 7.5; store frozen.
5. Water bath, 37°C.
6. Ethyl acetate.
7. Chloroform:methanol 95:5 (v/v).
8. Silica gel thin layer chromatography (TLC) plates and developing chamber.
9. X-ray film.

(b) Histochemical assay for CAT

1. PBS; PBS containing 10% fetal bovine serum and 1% bovine serum albumin.

2. Methanol: acetone 1:1 (v/v). Make fresh.
3. Rabbit anti-CAT IgG (5 Prime> 3 Prime, Inc., Paoli, PA).
4. Horseradish peroxidase-conjugated anti-rabbit IgG (ICN Biomedicals, Inc., Costa Mesa, CA).
5. *O*-dianisidine (Sigma).

36.3 METHODS

36.3.1 Transfection protocols

(a) Transient transfection

The methods below refer to polybrene-mediated transfection of the *Ae. albopictus* C7–10 cell line using the hsp-cat 1 plasmid. These protocols serve as a general guide to the transfection of other mosquito cell lines. In adapting this procedure to a new line, the parameters most likely to require optimization are the polybrene concentration (Note 5) and the conditions of post-transfection DMSO shock (Note 6).

1. On day 1, resuspend cells from an exponentially growing culture, take a cell count, and prepare sufficient E-5 culture medium for the number of plates to be transfected. Plate 4 mL (2.5×10^5 cells/mL) per 60 mm tissue culture plate (10^6 cells per plate).
2. Allow cells to attach overnight.
3. On day 2: Examine the cells under the inverted microscope. The cells should have resumed a normal morphological appearance and be uniformly distributed on the bottom of the dish, covering roughly 20 % of the total surface area.
4. To prepare the transfection medium, distribute serum-free medium (2.1 mL per plate to be transfected) into a glass beaker. For C7–10 cells, add polybrene (10 mg/mL) to the serum-free medium to a final concentration of 12.5 μg/mL. Mix thoroughly, then add DNA, to a final concentration of 1–12 μg/mL (Fallon, 1986) and mix again (Note 2).
5. When the transfection medium is ready, aspirate the E-5 medium from the cells. Using a pipette and taking care not to dislodge the cells, add 2 mL of freshly-prepared transfection medium to each plate. Return the plates to the 28°C incubator.
6. After 6–8 h, terminate the transfection by an optional 5 min treatment with DMSO/temperature shock. DMSO is added directly to the transfection medium to a final concentration of 10%, and the plates are placed in a 41°C incubator for 5 min (Mazzacano and Fallon, 1995; Note 6).
7. Remove the transfection medium by aspiration and re-feed the cells with standard growth medium, return the cells to a 28°C incubator (Note 3).
8. Monitor cell growth and, for transient assays, induce the hsp70 promoter (Note 1.a.) and measure expression of the foreign gene within 1–3 days. With the hsp-cat 1 construct in C7–10 cells, 45–70 h is an optimal period for incubation after transfection (Durbin and Fallon, 1985). By this time, the cells have grown to a near-confluent monolayer.

(b) Stable transfection

Various selectable marker genes have been used for stable transfection of mosquitoes or their cells. For example, the *neo* gene, which encodes resistance to G418, was used

in all three of the attempts to transform mosquito embryos (Miller *et al.*, 1987; McGrane *et al.*, 1988; Morris *et al.*, 1989). More recently, Monroe *et al.* (1992) have replaced the *neo* gene with the hygromycin phosphotransferase gene (*hyg*). Our own work with dominant selectable markers has focused on the *Ae. albopictus* dihydrofolate reductase gene (*dhfr*), which was cloned from methotrexate-resistant cells (Shotkoski and Fallon, 1993; Note 7).

1. Plate cells in 60 mm culture dishes as described above.
2. For stable transfection, add the foreign DNA of interest, together with an equimolar amount of plasmid encoding the selectable marker gene, at a final DNA concentration of 3–12 μg/mL, to serum-free medium containing polybrene as described above. Replace the growth medium with 2 mL of transfection medium.
3. After 8 h, remove the transfection medium and refeed the cells with E-5 medium.
4. After an additional 48 h, during which time transcription and translation of the selectable *dhfr* gene occur, add methotrexate to a final concentration of 1 μM.
5. Replace selective medium every 5 days until clones appear.
6. Once they have accumulated approximately 100 cells, clones are picked into plastic tissue culture tubes containing 2 mL of selective medium, and then expanded into flasks as the population increases. Store frozen populations or subclone as needed (Note 1).

36.3.2 Induction of the hsp70 promoter

1. For either transiently or stably transfected cells, transfer the plates from the 28°C incubator to a 41°C incubator for 2–6 h.
2. Return the cells to the 28°C incubator for a 2 h recovery period.
3. Collect the cells by centrifugation, wash with PBS, store the pellet frozen.

36.3.3 Assays for CAT activity

(a) Biochemical assay

1. Prepare radiolabelled substrate: Take from stock bottle, 0.1–1.0 μCi of ^{14}C-chloramphenicol for each sample to be assayed, plus one negative control (no enzyme) and one positive control (commercially available CAT enzyme); remove solvent by lyophilization. Dissolve isotope in 0.25 M Tris-HCl, pH 7.5 (10 μL per assay tube) and distribute 10 μL aliquots into tubes for the biochemical reaction.
2. On ice, sonicate the frozen cell pellet in 0.2 mL of 0.25 M Tris-HCl, pH 7.5.
3. Clarify the lysate by centrifugation at 10 000 rpm for 10 min.
4. Carefully transfer 150 μL of the supernatant to the corresponding reaction tube containing radiolabelled substrate; mix.
5. Initiate the reaction by addition of 20 μL of 4 mM acetyl CoA. Incubate 1 h at 37°C.
6. Terminate the reaction by extraction of the reaction mixture with 1.0 mL of ethyl acetate.
7. Separate the aqueous and organic phases by centrifugation.
8. Quantitatively remove the ethyl acetate (upper) organic phase to a new tube; evaporate the organic phase to dryness. This step may be done in a fume hood overnight.

9. Recover dried chloramphenicol and acetylated products in 30 μL ethyl acetate; vortex thoroughly.
10. Apply samples to silicagel TLC plates.
11. Develop TLC plates by ascending chromatography in chloroform: methanol (95:5). This step takes 2–3 h. The atmosphere in the developing tank should be vapour-saturated before use.
12. Allow plates to dry by evaporation; expose to X-ray film for 48–72 h.

(b) Histochemical assay for CAT

1. Heat-shock (2 h at 41°C, followed by 2 h at 28°C) the transfected cells.
2. Rinse the cells twice with PBS.
3. Fix the cells for 2 min using freshly-prepared methanol: acetone (1:1, vol/vol); rinse with PBS.
4. Make a serial dilution of rabbit anti-CAT antibody in sterile PBS containing 10% fetal bovine serum and 1% bovine serum albumin.
5. Test the dilution series to identify the highest dilution that gives a good signal with minimal background. Gerenday *et al.* (1989) found that a dilution of 1/250 to 1/500 was optimal, but this dilution is likely to vary with each lot of antiserum.
6. Incubate the rabbit anti-CAT (2 mL per 60 mm plate) with the fixed cells for 2–3 h at RT.
7. Remove the unbound antibody by washing the plate with four changes of PBS over 10 min.
8. Add appropriately diluted (see above) horseradish-peroxidase-conjugated anti–rabbit IgG (2 mL/plate); incubate 16 h at 4°C. Gerenday *et al.* (1989) used a 1/50 dilution of this second antiserum.
9. Remove the anti-rabbit IgG and wash the plates 4× with PBS.
10. Prepare a saturated solution of *o*-dianisidine by dissolving 135 mg in 7 mL of absolute ethanol. Before use, add 1 mL of saturated solution to 99 mL of PBS; then add 10 μL of 30% hydrogen peroxide. Add 2 mL of *o*-dianisidine/peroxide in PBS to each plate and incubate 1 h at RT. Check development of colour under the inverted microscope; photograph as desired.
11. Rinse plates thoroughly with ddH_2O. Dry and examine by light microscopy.

NOTES

1. Mosquito cell lines: There is no central repository for established insect cell lines, and cells are generally obtained from another lab in which they are in use. Many insect cell lines can be stored indefinitely under liquid nitrogen, in culture medium supplemented with serum to a final concentration of 20% and DMSO at a final concentration of 10%. We routinely purchase cell culture reagents from Sigma Chemical Co. (St. Louis, MO) and from GIBCO Laboratories (Grand Island, NY).

a. *Ae. albopictus* cell lines: C6–36 cells are featured in much of the work from the Arthropod-Borne and Infectious Disease Laboratory (AIDL) at Colorado State University (for a review, see Carlson *et al.*, 1995), while studies that originated in the laboratory of Victor Stollar at the University of Medicine and Dentistry of New Jersey make use of C7–10 and other derivatives of the LT C-7 line (Sarver

and Stollar, 1977). Both clones derive from the *Ae. albopictus* line of Singh (1967), are adapted to Eagle's (1959) medium containing various supplements (Sarver and Stollar, 1977; Igarashi *et al.*, 1977), and have been provided to various investigators upon request. Because the history of these lines may not be appreciated, the important details are summarized here.

Briefly, in an effort to ensure that his mosquito cells were free of latent dengue and Chikungunya viruses, Igarashi cloned cells which he adapted to Eagle's medium in the laboratory of Dr. Victor Stollar (Igarashi *et al.*, 1977) in the presence of antiserum to Chikungunya virus. When several clones were tested for ability to support replication of dengue and Chikungunya viruses, clone C6 gave the highest yield for every virus tested. The C6 cells were subcloned in the presence of a mixture of antisera to each of the four dengue virus types, yielding the C6–36 clone. Karyotype analysis showed that C6–36 cells were predominantly diploid, with a modal chromosome number of 6 (Igarashi, 1978). These cells have since been used in several laboratories, and have served as an important tool for testing expression of genes from Sindbis virus vectors (Carlson *et al.*, 1995). Although recent results of Monroe *et al.* (1992) suggest that the line has since become aneuploid, the biology of these cells *per se* has received little attention in the recent literature.

In contrast to the C6–36 cells, LT C-7 and its derivative, C7–10, were not exposed to anti-viral sera during the cloning process. Their cell biology has been examined extensively in the context of efforts to understand Sindbis virus-induced cytopathology. Over several years, these cells have maintained a relatively stable, diploid karyotype (Fallon and Stollar, 1987), although exceptions have been noted for various mutant lines (Mento and Stollar, 1978), particularly those resistant to methotrexate (Shotkoski and Fallon, 1990). In C7–10 cells and their derivatives, stable transfection gives copy numbers in the range of 100–500 per cell (Shotkoski and Fallon, 1993). In contrast, C6–36 cells sometimes maintain extremely high copy numbers of plasmid (up to 60 000), which occur in the form of double minutes and as large arrays that resemble full-sized chromosomes (Monroe *et al.*, 1992).

Finally, with *Ae. albopictus* C7–10 cells, we consistently observe modulation of temperature-inducible CAT expression from the *Drosophila* hsp70 promoter over a range of 34–41°C, with maximal induction at 41°C defined by the endogenous heat shock response in the C7–10 mosquito cells (Gerenday *et al.*, 1989). We note, however, that in C6–36 mosquito cells, an alternative construct containing the *Drosophila* hsp70 promoter is typically expressed constitutively (Monroe *et al.*, 1992). Whether this difference should be attributed to the source of the hsp-cat construct remains to be examined systematically.

The nature of these differences between two relatively closely-related lines from the same mosquito species has still to be thoroughly investigated, and it remains to be seen to what extent the variability may extend to the other mosquito cell lines that have been less extensively used in transfection studies.

b. Persistent infection with microbial agents: Mosquito cell lines may be persistently infected with viruses without exhibiting cytopathic effect. The potential presence of latent viruses in mosquito and other insect cell lines is a factor that must be considered if these cells are used as expression vectors to produce recombinant proteins of possible therapeutic value in vertebrates. Because many arthropod-borne viruses are cytopathic in vertebrate cells, the presence of such

viruses can sometimes be detected by inoculation of a vertebrate cell culture with medium from the insect cells. A particularly interesting example of such a latent viral infection is the Cell Fusing Agent (CFA), an RNA virus initially isolated from an *Ae. aegypti* cell line. The CFA genome has recently been sequenced and, based on homology studies, as well as its inability to replicate in vertebrate cell lines, Cammisa-Parks *et al.* (1992) suggest that CFA is the first example of an insect virus in the family Flaviviridae.

2. Culture medium: We maintain *Ae. albopictus* cells in Eagle's minimal essential medium (Eagle, 1959) containing glutamine, nonessential amino acids, antibiotics (antibiotics may be omitted), and fetal bovine serum at a final concentration of 5% (E-5 medium). The serum-free version of E-5 medium is called E-0. The details of the medium composition and its preparation have been described previously (Fallon, 1989). Although our cell lines also grow in various insect cell culture media that do not require bicarbonate buffer, we have not tested transfection conditions in these media. We note here that culture media designed for insect cells typically include serum and other supplements; the amount and nature of supplements may affect the concentration of polybrene required for efficient transfection. Undefined components, such as commercially available hydrolysates, can contribute unknown amounts of various precursors that might interfere with selection of stably transformed cells expressing dominant selectable marker genes, or with incorporation of a particular radiolabelled precursor. Adaptation of *Ae. aegypti* cells to E-5 medium is described by Lan and Fallon (1990). As far as we are aware, mosquito cell lines have not been adapted to serum-free media.
3. Routine maintenance of cells: C7–10 mosquito cells grow well at temperatures ranging from 28–34°C. In an air-conditioned building, water-jacketed incubators usually suffice. An incubator with cooling capacity may be required if ambient temperature is high and/or erratic. Note that the medium used in this laboratory is bicarbonate-buffered, thus cells are maintained under a 5% CO_2 atmosphere. To grow large quantities of cells in suspension culture for preparation of cellular DNA or RNA, we use glass culture jars equipped with side arms and a suspended stirring rod. The culture is seeded with 10^5 cells per mL, and cells attain a density of $2.5–5 \times 10^6$ per mL. Cells are maintained in suspension by stirring at low speed, and care should be taken that the stirring device does not generate excess heat in the incubator.
4. DNA: DNA for transfection is preferentially purified by caesium chloride centrifugation and made sterile by gentle mixing with chloroform (Fallon, 1989). Although early transfection studies with a variety of cell types considered the quality of the DNA of paramount importance, we have found that once parameters for transfection are established with a positive control such as hsp-cat 1, it may be convenient to use DNA prepared by simpler procedures. Miniprep procedures for small scale recovery of plasmid DNA typically yield material suitable for transfection, although we have not systematically compared the reproducibility of such material from preparation to preparation. Similarly, early transfection protocols with diverse cell types emphasized the importance of covalently closed DNA, but with C7–10 cells, DNA that has been linearized by restriction enzyme digestion is equally effective (Niu and Fallon, unpublished). We note, however, that with some other insect cell lines, linear DNA gives a low transfection frequency (Stiles *et al.*, 1992). Finally, precautions taken in our earli-

er work (Durbin and Fallon, 1985; Fallon, 1986), such as preparation of DNA in methylase-deficient bacteria, have proven unnecessary. For short-term (days to weeks) storage, DNA in 10 mM Tris-HCl, 1 mM EDTA, pH 7.5, should be held over a drop of chloroform at 4°C; we recommend frozen storage at –20 or –80°C over the long-term (months to years). DNA quality (conformation, size, freedom from contaminants) should be checked by agarose gel electrophoresis. Minor contamination with RNA after caesium chloride centrifugation does not interfere with the polybrene procedure, but its contribution to the apparent DNA concentration based on A_{260} should be considered (Fallon, 1986, 1989).

5. Other cell lines: The polybrene procedure described here has been successfully adapted to cells from the moth, *Spodoptera frugiperda*, which were maintained in Grace's medium containing fetal bovine serum. These studies included optimization of polybrene concentrations, which were about 10-fold higher than those used with mosquito cells. The details of this work are summarized by Helgen and Fallon (1990). It should further be noted that the DNA-polybrene combination is less toxic to cells than polybrene alone, and the polybrene procedure is somewhat flexible with respect to the relative amounts of DNA and polybrene (Fallon, 1986). A major drawback to polybrene-mediated transfection is the irreversible attachment of DNA to the surface of cells (Fallon, 1986). Only a small proportion of this DNA actually enters the cell, and it is only after stable transfection and subculture that membrane-bound, unincorporated DNA is diluted to the extent that chromosomal integration of foreign DNA can reliably be detected.
6. DMSO shock: With DNA constructs containing weak promoters, or untested heterologous promoters, it may be helpful to follow the transfection procedure with a DMSO shock (Mazzacano and Fallon, 1995). Treatment with DMSO or other membrane-active agents has been shown to enhance detection of foreign genes in many transfected cell systems (Aubin *et al.*, 1988; Walker, 1989). With mosquito cells, however, it is not known whether this DMSO treatment affects the number of cells that take up foreign DNA, or the number of DNA molecules taken up by an individual cell.
7. Constructs for stable transfection: For stable transfection, the foreign DNA of interest may be incorporated into an existing plasmid containing a second gene that will function as a dominant selectable marker. Alternatively, two (or more) independent constructs, only one of which contains a selectable gene, can be cotransfected simultaneously into a population of cells. The ability to incorporate foreign DNA by transfection is commonly called 'competence', and it has been generally observed that a competent cell takes up all DNA species to which it is exposed. In our own experience with mosquito cells, this has consistently been found to be the case. The selectable marker constructs used in this laboratory contain the complete coding region for the dihydrofolate reductase (*dhfr*) gene, including the single 56 bp intron and upstream DNA containing all of the known transcription initiation sites (Park and Fallon, 1993). A particularly versatile construct for stable transfection is the plasmid called pDHFR:cat-1, which contains both the hsp70 promoter-*cat* gene fusion from Di Nocera and Dawid's hsp-cat 1, and the mosquito *dhfr* gene under the regulation of its native promoter. For cotransfection, we have also described use of a smaller plasmid, pFSDHFR9, which contains only the selectable mosquito *dhfr* gene (Shotkoski and Fallon, 1993).

8. Applications of stable transfection in mosquito cells: In one of the first applications of cotransfection to *Ae. albopictus* cells, we have used the *Drosophila* hsp70 promoter to express the *dhfr* gene in sense and antisense orientations. Here, as anticipated, temperature induction of antisense *dhfr* mRNA substantially reduced the plating efficiency and survival of transfected mosquito cells (Shotkoski and Fallon, 1994). In a more recent application, Mazzacano and Fallon (1995) used thymidine kinase-deficient (TK^-) mosquito cells as recipients for the *tk* gene from herpes simplex virus (HSV). Here, the TK^- cells were cotransfected with a construct containing the viral *tk* gene and with pDHFR:cat-1. After a 48 h interval to allow accumulation of DHFR enzyme, transformants were selected in 1 μM methotrexate. Clones thus obtained were grown into populations, which were shown to be positive for expression of temperature-inducible CAT activity. The populations were then subjected to a second selective medium containing both methotrexate and azaserine, to ensure that only TK^+ cells expressing the viral gene would survive. In this example, the combined use of transfection and somatic cell mutants allows the analysis of pathways that would be difficult to dissect at the level of the transformed organism. Thus, transfection of cells in culture will continue to play a role in mosquito transformation efforts even after the anticipated embryo transformation is accomplished.

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CHAPTER THIRTY SEVEN

Viral transducing vectors for mosquitoes

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37.1 INTRODUCTION

We define transduction as the process by which a gene of interest is packaged into virus particles and delivered to cells by the infectious process. A number of different animal viruses have been developed as transducing vectors for vertebrate cells and organisms. Considerable emphasis has been placed on retroviruses, adenoviruses, parvoviruses and poxviruses to develop novel vectors for applications such as gene therapy and immunization. However, with the exception of the baculoviruses of lepidopterans (Luckow and Summers, 1988), little has been done to develop gene transfer vectors for insects. One reason for this may be the relative scarcity of well-characterized DNA viruses of insects. Recently, two viruses have been adapted for introduction of genes into mosquito cells and they have the potential for transduction of genes into whole mosquitoes (Carlson *et al.*, 1995). These are: (i) the densonucleosis virus of *Aedes aegypti* (AeDNV); and (ii) pantropic retroviruses pseudotyped with the vesicular stomatitis virus glycoprotein. These two systems are quite different in their biology and will be treated separately in this chapter.

37.1.1 *Aedes* densonucleosis virus vector

The AeDNV was isolated originally from a laboratory colony of *Aedes aegypti* mosquitoes (Lebedeva *et al.*, 1973). The virus orally infects mosquito larvae of the genera *Aedes*, *Culex* and *Culiseta*. It is pathogenic to both larvae and adults (Buchatsky, 1989). The virus can be detected in almost all tissues of infected mosquitoes and produces hypertrophy of affected cells, as well as characteristic dense Feulgen-positive nuclear inclusions. Recently, a closely related virus has been isolated from persistently infected *Ae. albopictus* cells (Jousset *et al.*, 1993; Boublik *et al.*, 1994).

Densonucleosis viruses, or densoviruses, are parvoviruses of arthropods. As with all parvoviruses, the viral genome is a linear single-stranded DNA molecule with short double-stranded hairpin ends that are necessary for replication and packaging. Densovirus particles are nonenveloped icosahedral particles that are relatively resis-

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tant to heat and chemical insults. The genome of the AeDNV is about 4000 nucleotides in length (Afanasiev *et al.*, 1991). The left side of the genome contains two overlapping open translational reading frames encoding two nonstructural proteins, NS1 and NS2 (Figure 37.1). NS1 is believed to be necessary for replication and packaging of the viral DNA and for transactivation of gene expression from the viral promoters. The function of NS2 is unknown. The right side of the genome contains the gene that encodes the viral capsid proteins VP. The nonstructural proteins are expressed from a promoter P7 located at map unit 7 (the genome is 100 map units long). The capsid proteins are expressed from a promoter P61 at map unit 61. A stable stem and loop secondary structure was observed in the sequence located between the p7 promoter and the NS2 initiation codon (5′-end of putative mRNA coding for NS1 and NS2) (SL in Figure 37.1). This region was shown to be crucial for expression from the AeDNV p7 promoter. The AeDNV capsid proteins contain a nuclear targeting sequence on the N-terminus (NTS in Figure 37.1). The sequence is used to transfer the capsid proteins to nuclei after they are synthesized in cytoplasm. It has been shown that if a foreign gene is fused to this sequence the resulting recombinant protein is also delivered into nuclei (Afanasiev *et al.*, 1994).

Early in the infection the single-stranded viral genomic DNA is converted to a double-stranded replicative form (RF) DNA by extension of the 3′ hairpin end of the genome by cellular DNA polymerases. This RF DNA is replicated to produce progeny viral genomes and transcribed by cellular RNA polymerase to produce used mRNA for synthesis of the proteins NS1, NS2 and VP. NS1 is necessary for nicking and unwinding double-stranded RF DNA to initiate packaging of the single-stranded progeny genomes into preassembled empty capsids made of VP.

The development of gene transfer vectors based upon the AeDNV was made possible by the construction of an infectious clone of the viral genome. The plasmid pUCA (Afanasiev *et al.*, 1994) contains a full length double-stranded copy of the AeDNV genome. When pUCA DNA is transfected into *Ae. albopictus* (C6/36) cells, the genome is cleaved out of the plasmid and a productive infection begins. The use of AeDNV to transduce foreign genes is possible because the *cis*-acting elements necessary for replication and packaging are contained in the ends of the genome. The viral protein NS1, NS2 and VP can all be provided *in trans*.

In order to package a gene of interest into virus particles, it must be cloned between the ends of the genome in pUCA. All of the coding sequences for NS1, NS2 and VP can be replaced with foreign DNA, provided the sequences that form the hairpin ends of the single-stranded genome are left intact. Since the AeDNV capsids are icosahedral and of fixed volume, transducing genomes that are appreciably greater (more than about 15%) than unit length (ca. 4000 bp) are not efficiently packaged. Consequently a gene < 4000 bp coding for a protein of about 150 000 Da can be packaged into AeDNV transducing particles. When a plasmid containing the transducing genome is cotransfected into C6/36 cells with pUCA as a helper, the viral proteins expressed from pUCA mediate excision, replication and packaging of the transducing genome into AeDNV particles. Thus, these cotransfected cells produce both infectious AeDNV and recombinant transducing particles. If the transducing virus is cotransfected with a helper construct that is incapable of replication (e.g. lacking one or both of the terminal hairpin sequences) but still expresses the viral proteins, only transducing virus is produced (Figure 37.2). A gene of interest can be expressed from the transducing AeDNV genome as a fusion or nonfusion protein. In the first case, the DNA sequence should be cloned into the AeDNV genome to be in frame with the amino-acid

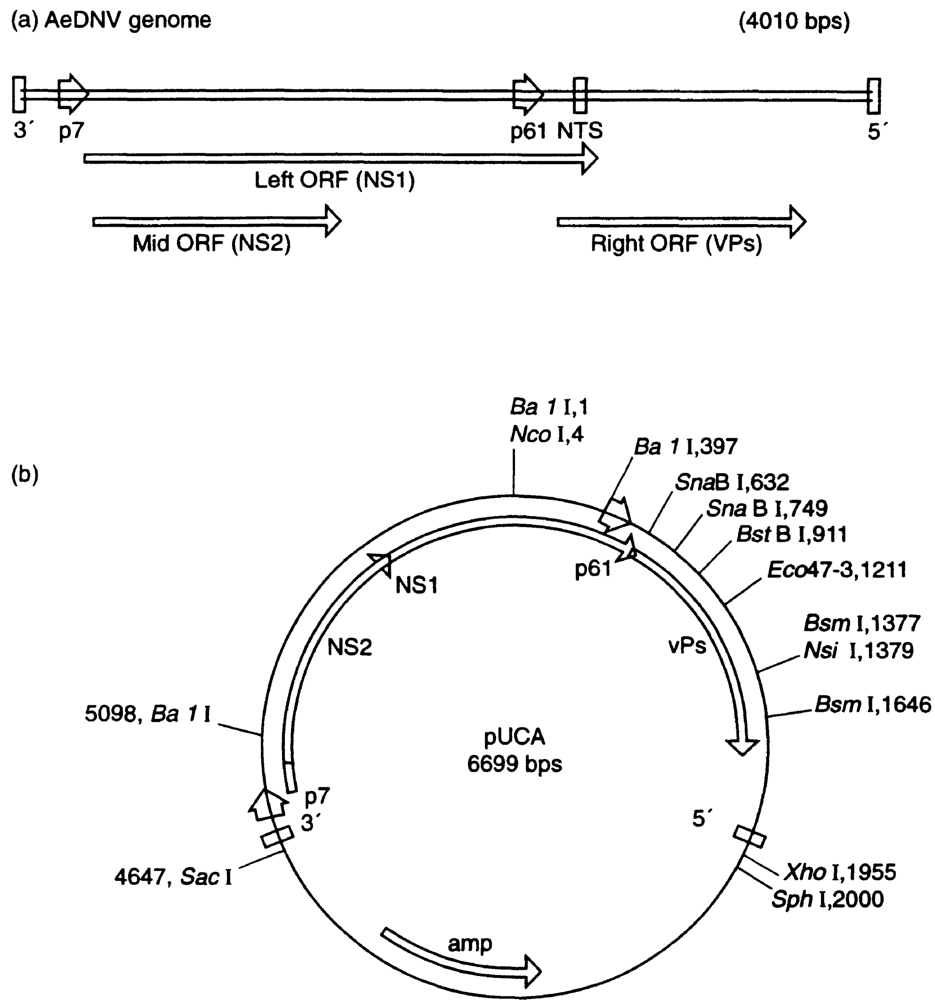


Figure 37.1 Genome structure of AeDNV (A) and the map of pUCA plasmid (B). 3′ and 5′ – self-complementary sequences which can assume Y-shaped secondary structures (necessary for replication and packaging); p7 and p61 – promoter sequences driving expression of the nonstructural and virion proteins of AeDNV, respectively; NTS – nuclear targeting signal sequence observed on the N-terminus of the virion proteins. On the map several restriction enzyme sites are shown which are unique or as *BalI* (= *MscI*) can be used for 'shortening' of the AeDNV recombinant genome.

sequences of the virus polypeptides and downstream of the corresponding initiation codons. The product of the expression will be chimeric and besides the sequence of a foreign gene, will contain a small portion of the N-terminus of the AeDNV polypeptides. In many cases it has been shown that such fusion proteins preserve the functions of the native polypeptide.

We have found that foreign proteins can be efficiently expressed from both p7 or p61 promoters fused into any of the three AeDNV ORFs (Figure 37.1). Expression of reporter genes (β-galactosidase and β-glucuronidase) showed that the maximum efficiency could be attained when the reporter genes had been fused into the left

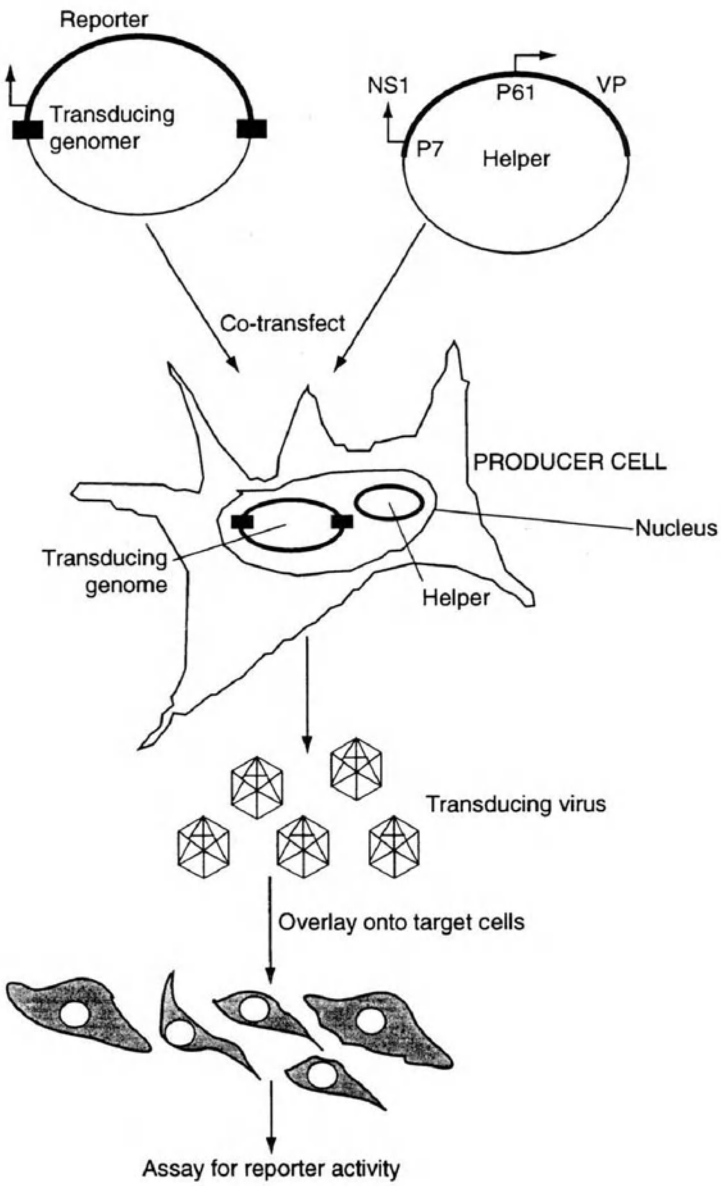


Figure 37.2 Scheme of recombinant transducing particles production. A plasmid carrying a gene of interest (reporter) cloned into AeDNV (transducing) genome is cotransfected with a helper plasmid into mosquito (producer) cells. The helper plasmid provides the nonstructural (NS1) and virion (VPs) proteins. NS1 is needed for rescuing the transducing genome from the plasmid and its subsequent replication; VPs are needed for encapsidation of the genome into transducing particles (virus). Self-complementary terminal sequences of the AeDNV genome are represented with black rectangles.

ORF. If a gene of interest must be expressed from the AeDNV promoters as a nonfusion protein, efficient expression can be achieved from p61. In this case, if a gene of interest is long (which makes the recombinant genome >4400 bp), the sequences located between p7 and p61 promoters must be eliminated (using restriction enzymes). Another possibility is to clone a foreign gene into the AeDNV genome but express it from other inserted promoters. In this case, the gene of interest with the

promoter sequence should be cloned into the AeDNV genome to preserve only the terminal sequences (about 200 bp from both ends). Recombinant virus produced by these cotransfected cells can then be used to infect fresh mosquito tissue culture cell or mosquito larvae.

37.1.2 Pantropic retroviral vectors

Retroviruses have a RNA genome packaged in a nucleocapsid surrounded by an envelope derived from the host cytoplasmic membrane. Retroviruses are unique among RNA viruses because the genome cycles between RNA and DNA forms during the infectious cycle. Early in the infection the RNA genome is converted to a double-stranded cDNA by RNA-dependent DNA polymerase (reverse transcriptase) carried in the virus particle. This double-stranded cDNA is subsequently integrated into the host genome by the virus-associated integrase to form a provirus. Messenger RNA's for the viral proteins (*gag*, *pol* and *env*) and full length RNA genomes are transcribed from this provirus. New virus particles are assembled from these components at the cellular surface, and bud from the plasma membrane.

Retroviruses are attractive as gene transfer vectors because of their ability to stably integrate into the host genome. To convert a retrovirus into a retroviral vector, the viral-specific coding region bounded by the long terminal repeat (LTR) sequences is removed and replaced by genes and promoters of interest. Vectors are assembled in packaging cell lines that stably express viral-specific proteins. Many of the commonly used retrovirus vectors are based on the Moloney murine leukaemia virus (MoMLV).

Previous limitations to the use of MoMLV-derived retroviral vectors as a tool for gene transfer in insects included the inability to obtain high titre stocks and the narrow host cell range of these vectors. To overcome these disadvantages, a new class of pantropic retroviral vector was developed that can be concentrated to high titre and has a broadened host cell range.

Pseudotyped pantropic vectors contain the vesicular stomatitis virus G glycoprotein (VSV-G) substituted for the retroviral envelope protein (Burns *et al.*, 1993). The host range of the amphotropic and ecotropic retroviral vectors is restricted to mammalian cells that express a specific protein receptor on the cell surface that acts as a ligand for the envelope protein. The VSV-G envelope protein, in contrast, binds to phospholipid components in the cell membrane, thus permitting these VSV-G pseudotyped particles to enter virtually every cell with a plasma membrane (Mastromarino *et al.*, 1987). The extremely broad host cell range of these vectors has been demonstrated by infection of a wide variety of organisms including fish, newts, marine invertebrates and insects (Lin *et al.*, 1994; Burns *et al.*, 1994; Lu *et al.*, 1996; Matsubara *et al.*, 1996).

An additional consequence of the substitution of VSV-G for the retroviral envelope protein is increased stability of the virus particle. Pantropic vectors can be concentrated by simple physical methods such as ultracentrifugation to high titre (10^8–10^9 cfu/mL) with greater than 75% recovery of the total number of infectious particles (Burns *et al.*, 1993). This method allows generation of vector stocks at titres sufficient for microinjection of nanolitre volumes into insect embryos. Transgenic zebrafish have been created by microinjection of concentrated vector into the developing embryo (Lin *et al.*, 1994).

(a) Advantages of pantropic vectors

The distinct advantage of retroviral vectors over other transducing viruses is the ability of the provirus (reverse transcribed RNA genome) to stably integrate into the host cell genome. Vectors can accommodate up to 10–13 kb of heterologous sequence including internal promoters and coding regions. Unlike the mobile genetic elements of insects, the integrated proviral DNA does not translocate in the host cell genome. Nor can it result in the production of infectious particles since it carries no viral-specific coding sequences.

Retroviral stocks are extremely stable and can be stored at least 4 months at –70°C without loss of infectivity. In addition, the vectors are stable under a variety of environmental conditions including incubation at RT, at high osmolarity (800 mOsm), and high pH (pH 8.5). Acidic conditions (pH <4.0) are, however, detrimental to the virus and rapidly decrease the viral titre.

(b) Disadvantages of pantropic vectors

Pantropic retroviral vectors are infectious to all mammalian cells, including human cells. Thus, during the production of vector stocks, care must be exercised in the handling of these viruses to prevent inadvertent human infection. Retroviral vectors derived from the murine leukaemia viruses have been approved for use in human gene therapy and have been administered to hundreds of patients with no untoward effects. Laboratory practices consistent with Biosafety Level 2 (BSL-2) recommendations are considered adequate for containment of the virus during production of both concentrated and unconcentrated stocks. These practices require only the equipment and facilities necessary for mammalian tissue culture and will be highlighted in the following protocol.

37.2 MATERIALS

37.2.1 *Aedes* densonucleosis virus vector

1. AeDNV expression system:
   a. pUCA plasmid (INVITROGEN, San Francisco, California). The full-length AeDNV genome cloned into pUC 19. This plasmid is used to clone a gene of interest and express it as a fusion or nonfusion protein from either of the AeDNV promoters (p7 or p61), or any heterologous promoters. The sequence and the map of the plasmid, including convenient cloning sites, can be obtained from INVITROGEN. All cloning protocols can be found in Sambrook *et al.* (1989).
   b. pUCAΔ5′ helper plasmid. The pUCA plasmid in which the right terminus of the AeDNV genome has been eliminated, making it incapable for excision and hence replication and packaging. This plasmid is used to supply the AeDNV nonstructural and capsid proteins.
2. *Aedes albopictus* (C6/36) cells (Igarashi, 1978). These cells have been used to produce recombinant transducing particles though AeDNV infects also *Ae. aegypti* (ATC10) and *Anopheles gambiae* (MOS-55) cells (Marhoul and Pudney, 1972). C6/36 cells are grown at 28°C in Leibovitz's L–15 medium supplemented with 10% fetal bovine serum and antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin).

3. Leibovitz's L-15 medium (GibcoBRL, Grand Island, NY).
4. PBS (137 mM NaCl, 2.6 mM KCl, 1.5 mM Na_2HPO_4, 8.1 mM KH_2PO_4, pH 7.5).
5. Lipofectin reagent (GibcoBRL).
6. Mosquito larvae are fed on alevin (200 mg daily) and reared at 28°C in 2 L pans.

37.2.2 Pantropic retroviral vectors

(a) Cell lines, media and equipment for tissue culture

1. PA317 mouse fibroblasts (Miller and Buttimore, 1986), 293 gag-pol human kidney epithelial cells (Burns *et al.*, 1993), and 208F rat fibroblasts (Quade, 1979) are grown in Dulbecco's Modified Eagle Medium (DMEM) with 4.5 g/L glucose and supplemented with 10% fetal calf serum, penicillin (100 U/mL), streptomycin (100 μg/mL), and L-glutamine (2 mM).
2. Disposable, sterile plasticware: syringe tip filters (0.45 μm), 10 cm tissue culture dishes, 6-well tissue culture dishes, 48-well tissue culture dishes, 1 mL and 10 mL pipettes, micropipettor tips, 50 mL and 15 mL polypropylene screw-top tubes, freezing vials.
3. Incubator: 10% CO_2, 37°C.

(b) Reagents for cell culture and concentration of pantropic vector

1. Polybrene stock solution (4 mg/mL): dissolve 0.4 g of hexadimethrine bromide in 100 mL of sterile water and store at 4°C. Aliquoted stocks can be stored at –20°C for prolonged periods.
2. G418 stock solution (40 mg/mL): dissolve 4 g active Geneticin (GibcoBRL) in 100 mL 0.1 M Hepes, pH 7.9, filter sterilize (0.45 μm) and store at 4°C. Aliquoted stocks can be stored at –20°C for prolonged periods (Notes).
3. Hygromycin B stock solution (40 mg/mL): dissolve 1×10^6 U active Hygromycin B in 25 mL sterile ddH_2O, filter sterilize (0.45 μm), and store at 4°C. Aliquoted stocks can be stored frozen at –20°C for prolonged periods.
4. Phosphate buffered saline (PBS): 137 mM NaCl, 2.6 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4. Autoclave and store at RT.
5. 0.1× Hank's Balanced Salt Solution (HBSS): Dilute 1 mL HBSS in 9 mL sterile ddH_2O and store at 4°C.
6. Giemsa stain.
7. Reagent grade methanol.
8. Transfection reagents for calcium phosphate co-precipitation of plasmid DNA (Graham and van der Eb, 1973).

(c) Reagents for PCR detection of proviral LTR in infected cells

1. DNA extraction buffer: 100 mM NaCl, 10 mM Tris-HCl, pH 7.4, 25 mM EDTA, 0.5% SDS, and 200 μg/mL proteinase K. Store buffer at 4°C and add proteinase K (20 mg/mL frozen stock) just before use.
2. 10× *Taq* buffer: 500 mM KCl, 100 mM Tris-HCl, pH 8.4, 15 mM $MgCl_2$. Store at 4°C.
3. PCR primers for amplification of the proviral LTR: dilute to a final concentration of 12.5 μM. Use 1 μL each primer/reaction.

Upstream primer sequence for the MoMLV LTR:
5′-AGGACCTGAAATGACCCTGT-3′
Downstream primer sequence for the MoMLV LTR:
5′-ACGGGTAGTCAATCACTCAG-3′

4. 10× dNTP stock solution: dilute dCTP, dUTP, dATP, and dGTP with ddH_2O to a final concentration of 2 mM. Store at 4°C.
5. *Taq* polymerase.

37.3 METHODS

37.3.1 *Aedes* densonucleosis virus vectors

(a) Production of transducing particles

Transfection of C6/36 (and, we believe, any other mosquito cells with little adjustment) with plasmids can be performed using the lipofectin reagent according to the manufacturer's specifications. The following procedure was developed for 75 mL cell culture flasks with 10–15 mL of medium. Plasmid DNA for transfection should be purified by two equilibrium centrifugations in caesium chloride–ethidium bromide density gradients (Sambrook *et al.*, 1989), extracted at least 3× with water-saturated *n*-butanol, and precipitated with 2 vol of ethanol. The pellet is rinsed with 70% ethanol and dissolved in 0.4 mL of distilled water. Ammonium acetate is added to give a final concentration of 2 M and DNA is precipitated again with 2 vol of ethanol. The pellet is rinsed with 70% ethanol and dissolved in sterile deionized water at the concentration 200 μg/mL.

1. Cells are seeded 24 h prior to transfection at a density that will result in 50% confluent monolayer at the time of transfection.
2. Next day a lipofectin-DNA complex is made by mixing in a polystyrene tube:
   8.5 mL Serum-free L-15 medium
   150 μL (=30 μg) Plasmid pUCA with a gene of interest inserted
   300 μL (=60 μg) Helper plasmid pUCAΔ5′
   150 μL Lipofectin reagent
3. Incubate the mixture for 15 min at RT. During the incubation rinse the flask of cells to be transformed 3× with sterile PBS (or L-15 medium).
4. The final PBS rinse is removed and replaced with 9 mL of the Lipofectin-DNA complex.

Following incubation for 8 h at 28°C (or RT), the transfection medium is replaced with L-15 supplemented with 10% of fetal bovine serum and antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin). From 2–4 days after transfection, when cells recover, undergo division and reach approximately 100% monolayer, transducing particles can be harvested.

(b) Transduction of a gene of interest into mosquito cells

If the gene of interest is to be delivered to fresh cells in a cell culture the following procedure can be used:

1. Scrape the transfected producer cells with a rubber policeman and transfer the suspension of cells to a sterile plastic tube with a cap.

2. Pellet cells by spinning at 1500 **g**, 10 min, RT, and save the culture medium supernatant (which might contain up to 50% of transducing the particles).
3. Resuspend pellet (by vigorous vortexing) in 200 μL of sterile water.
4. Freeze cells in –20 to –70°C freezer (or liquid nitrogen).
5. Thaw cells in 37°C water bath.
6. Repeat freezing and thawing twice more.
7. Centrifuge the suspension 1500 **g** at RT for 10 min.
8. Save the supernatant.

This supernatant contains about 50% of all transducing particles generated, and can be used directly by adding into the medium of fresh cells or otherwise mixing with the supernatant saved on step 2. It is important to keep the volume of the transducing medium to a minimum to attain the highest ratio of transducing particles per cell. Using *LacZ* gene of *E. coli* (as the reporter gene) fused into mid ORF and expressed from the p7 AeDNV promoter, we have detected the β-galactosidase expression in 20% of transduced cells.

(c) Transduction of a gene of interest into mosquito larvae

If a gene of interest is to be transduced into mosquito larvae a method of efficient infection described recently by Barreau *et al.* (1994). Larvae are placed in a culture flask with the transfected transducer cells where larvae acquire the transducing particles, not only by infection from the medium, but also by feeding on cells containing the particles:

1. Hatch larvae about 15 h before transduction.
2. Scrape cells in a flask with transducing particles.
3. Using a Pasteur pipette transfer larvae (first instar) in minimal volume to this flask (approx. 250 or more per 75 cm^2 flask containing 10–15 mL of medium).
4. Keep the flask horizontal with the cap loosely screwed in a chamber at 28°C.
5. After 48 h the larvae (which have already moulted to the second instar) are transferred into 2 L pan filled with 1 L of distilled water.
6. Rear larvae at 28°C for optimal time to observe the effect of a transduced gene expressed.

37.3.2 Pantropic retroviral vectors

(a) Production of pantropic vector

Pantropic vectors can be produced with a wide variety of genes and internal promoters. Described here is the production of the pantropic vector LN-hsp70-hyg-L in which the neomycin phosphotransferase gene (N) is expressed from the 5′ MoMLV LTR (L) and the hygromycin phosphotransferase gene (hyg) is expressed from the *Drosophila melanogaster* heat shock promoter (hsp70) (Figure 37.3). The LTR is not functional in insect cells. Thus, selection of transduced insect cells with this construct is accomplished with the antibiotic hygromycin B. The mammalian producer cells can be selected with either G418 or hygromycin B. Since the hsp70 is a weak, constitutive promoter in mammalian cells, selection with G418 is preferred for the mammalian cells. The retroviral genome can be reconfigured by molecular cloning to create vectors that express selectable markers such as β-galactosidase (Matsubara *et al.*, 1996), luciferase, and chloramphenicol acetyltransferase under the control of different promoters. The retroviral construct, LN-hsp70-hyg-L, can be detected in infected insect cells by ampli-

fication of proviral-specific sequences, or by Southern hybridization of hygromycin-resistant colonies probed with labelled retroviral sequence from the plasmid used to create the packaging cells.

The general method for production of pantropic virus is illustrated in Figure 37.4. In the first step, amphotropic virus is produced in PA317 cells. In the second step, this virus is used to infect 293 cells stably expressing the MoMLV *gag* and *pol* genes. Pantropic virus particles are produced when the 293 gag-pol/LN-hsp70-hyg-L cells are transfected with a plasmid expressing the VSV-G protein under the control of a strong promoter (human cytomegalovirus, hCMV).

All work with pantropic retroviral vectors is performed according to BSL-2 practices. It should be remembered that while pantropic vectors are replication incompetent, they readily infect a broad range of cells including all dividing human cells. Care must be taken to avoid creating aerosols of the virus except inside a tissue culture hood equipped with a HEPA filter.

(b) Generation of stable 293 gag-pol producer cell line

1. Day 1: Seed approximately 1–5 × 10^5 PA317 cells in a 10 cm tissue culture plate.
2. Day 2: Transfect by standard calcium phosphate co-precipitation (Graham and van der Eb, 1973) with 20 μg pLN-hsp70-hyg-L.
3. Day 3: Seed approximately 1 × 10^5 293 gag-pol cells in a 10 cm tissue culture plate for infection on day 4.
4. Day 4: Aspirate and filter (0.45 μm) supernatant from PA317 cells transfected on day 2. Use 1–2 mL to infect 293 gag-pol cells in the presence of 8 μg/mL polybrene (Notes).
5. Day 5: At least 16 h after infection, aspirate culture medium from infected 293 gag-pol cells and replace with fresh medium containing 400 μg/mL of G418. Continue to re-feed culture with fresh medium containing G418 until resistant colonies appear in 12–14 days. Since 293 gag–pol cells adhere only loosely to the tissue culture plate, care must be taken to avoid disturbing the colonies when changing the medium.
6. Week 2: Aspirate supernatant. Pool G418-resistant colonies by resuspending cells in G418-containing medium. These cells are referred to as 'producer cells' (Notes).

(c) Generation of pantropic vector

1. Day 1: To generate pantropic vector, seed a 10 cm plate with 1–5 × 10^5 pooled producer cells.
2. Day 2: Transfect these cells by calcium phosphate co-precipitation with 20 μg of the plasmid hCMV-VSV-G. Remove the calcium phosphate-DNA precipitate after 6–8 h and gently replace medium. Care must be taken not to disturb the monolayer, as the cells are fragile and poorly adherent after transfection. Longer incubation with the precipitate will kill the cells.
3. Day 3: Seed a 10 cm plate with 1 × 10^5 208F rat fibroblasts for titring virus.

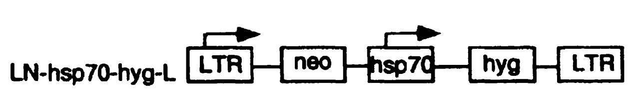


Figure 37.3 Genetic structure of vector LN-hsp70-hyg-L.

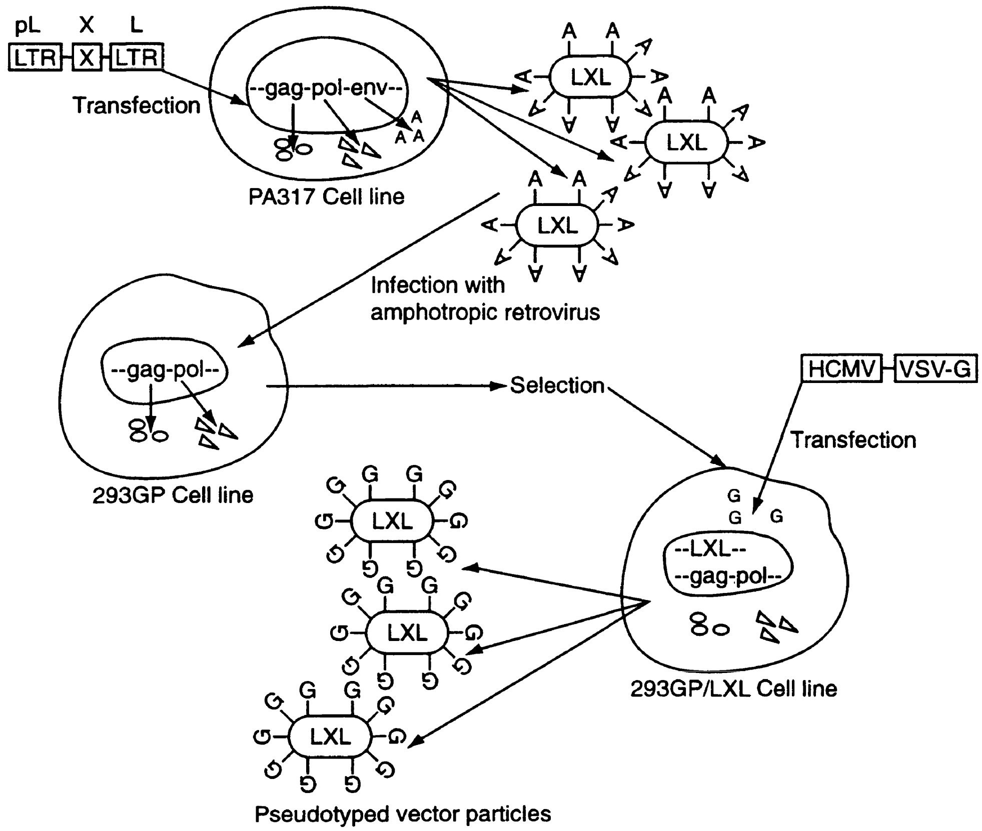


Figure 37.4 Method for production of pantropic retroviral vectors.

4. Days 4–6: Collect virus-containing culture supernatant once a day beginning 48 h post-transfection. The highest titre virus is produced between 48–96 h post-transfection. Culture supernatant should be filtered (0.45 μm) and an aliquot removed to determine virus titre. Remaining supernatant may be frozen at –70°C until needed.
5. To titre virus, dilute appropriately with DMEM (e.g. anticipate 1×10^6 cfu/mL, use 20 μL of 1:1000 dilution of virus per 10 cm plate; at this dilution, one colony on the plate represents a titre of 5×10^4 cfu/mL). Add 10 μL of polybrene stock to 208F cells (final concentration in 10 mL of medium will be 8 μg/mL). Add diluted virus to 208F cells.
6. After overnight incubation with virus and polybrene, replace medium with DMEM complete medium containing G418 (400 μg/mL). Replace medium every 3–4 days until colonies are visible (usually 10–14 days).
7. Fix colonies in methanol and stain with Giemsa stain to facilitate counting colonies. Calculate titre of virus. Assume that each colony represents one infectious unit of virus.

(d) Concentration of pantropic vector

1. UV sterilize swinging buckets, caps, and ultracentrifuge tubes for an SW41 or other suitable swinging bucket rotor (e.g. SW 50.1 [5 mL], SW 28 [37 mL]).

2. Under sterile conditions, transfer viral culture supernatant to the centrifuge tubes and tighten caps.
3. To pellet virus particles, centrifuge at 50 000 g (25 000 rpm in an SW41 rotor), 4°C, 90 min. Decant supernatant and wipe inside walls of tube with a tissue (nonsterile Kimwipe works well) to remove excess medium. Place tube upright in ice and add 30 μL 0.1× HBSS/tube. Allow invisible pellet to resuspend undisturbed at 4°C overnight.
4. The day following centrifugation, resuspend pellet by pipetting (with a P200 pipettor) vigorously up and down, but avoid foaming the solution. Concentrated vectors stocks may be frozen at –70°C for at least 4 months without loss of titre.

(e) Infection of insect cells

1. Prepare monolayer of insect cells to be infected. Add polybrene to final concentration of 2–8 μg/mL. Add virus. The volume of viral supernatant to be added depends on density of cells, titre of virus, and infection efficiency. For C6/36 cells growing in a 10 cm plate, add 7–10 mL of virus with a titre of 1×10^6 cfu/mL to obtain approximately 100 hygromycin-resistant colonies.
2. After overnight incubation, replace medium with standard medium for cell line.
3. At 48 h post-infection, replace medium with medium containing hygromycin B (100 μg/mL). The optimal concentration of hygromycin B may vary, and killing curves should be established independently for each different insect cell line. The optimal concentration of hygromycin B is one that kills >80% of the cells in 5–7 days.

(f) Detection of provirus in infected insect cells

A polymerase chain reaction (PCR) assay can be used to detect provirus in infected insect cells. A positive PCR signal indicates that attachment, uncoating, and reverse transcription of the RNA genome into the DNA provirus has successfully occurred. It should be noted that the following assay detects infected cells containing proviral DNA but does not provide information about integration of the provirus into the host cell genome.

1. Lyse approximately 1×10^5 cells infected 48–72 h previously with the pantropic vector in 500 μL DNA extraction buffer at 37°C overnight.
2. Isolate the DNA following phenol extraction and isopropanol precipitation and resuspend in 25–100 μL sterile ddH_2O.
3. Measure the concentration of DNA spectrophotometrically and use 0.5–1 μg DNA per PCR reaction.
4. Add the DNA to a 500 μL microcentrifuge tube containing 50 μL of mineral oil. Centrifuge briefly and heat at 100°C for 2 min. Quickly chill on ice.
5. Prepare the PCR reaction mix (25 μL final volume) by mixing 0.5 μM each primer (1 μL each of 12.5 μM stock), 200 μM dNTP's (1 μL of 2 mM stock), 2 U *Taq* DNA polymerase, 2.5 μL 10× *Taq* buffer, and sufficient water to equal 25 μL when mixed with the DNA. Chill reaction mix on ice and add to DNA by centrifuging briefly so that reaction components are below mineral oil overlay.
6. Place the reaction tube in a thermal cycling device and proceed with the following thermal profile: 94°C for 3 min, followed by 40 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. End with a final extension step at 72°C for 5 min.

7. Analyse 10 μL of PCR reaction mixture on a 2% agarose gel stained with ethidium bromide. If additional sensitivity is required, the resolved DNA can be transferred to a nylon membrane and hybridized with a labelled LTR PCR product probe to visualize the predicted 244 bp amplification product.

NOTES

Aedes densonucleosis virus vector

1. pUCA plasmid can be used as the helper plasmid instead of pUCAΔ5′. In this case, expect that wild type virus particles will be generated at a higher frequency than recombinant transducing particles.
2. Purification of plasmid DNA by two times CsCl gradient centrifugation is important because after the first gradient the DNA still contain different amount of RNA which might not only be transfected to cells but make it more difficult to determine the concentration of a plasmid.
3. LipofectAMINE reagent (GibcoBRL) can be used instead of Lipofectin. For the procedure described above we used 30 μL of LipofectAMINE.
4. Time of transfection can be varied. It is important to remember that in order to generate the maximum titres of transducing particles the following conditions should be optimized in each separate case:
   a. density of cell at the moment of transfection
   b. ratio of transducing: helper plasmids
   c. ratio of DNA: lipofectin reagent
   d. time of transfection, which depends on (a) and (c)
   e. time when transducing particles are harvested post-transfection.

   This is important because the generating recombinant particles might reinfect cells in the primary culture and be lost from the pool. Optimization could be performed using a reporter gene (e.g. LacZ gene of *E. coli*) cloned into pUCA plasmid (INVITROGEN, San Francisco, CA). There is no detectable endogenous β-galactosidase activity in C6/36 cells. For β-galactosidase detection in mosquito cells using Xgal staining or measuring it by ONPG cleavage assay see Afanasiev *et al.* (1994).
5. Transducing particles should be added to cell culture or living mosquito larvae in a minimal volume in order to keep the concentration of the particles per cell (or larvae) at a maximum.
6. For hatching larvae, different synchronization techniques can be used. If baker's yeast is used it is important to remove it afterwards by washing the larvae 2–3 times in tap water.
7. When choosing a foreign gene to be transduced into mosquitoes one should remember that mosquito cells might express a gene with the same or similar functional characteristics (so called endogenous activity). Activities similar to bacterial β-galactosidase and β-glucuronidase have been detected at least in some tissues of mosquitoes.

Pantropic retroviral vector

1. Pay careful attention to the calculations for the reconstitution of G418 to ensure that the final concentration represents 100% active drug. Commercially available

products usually contain only 60–70% active compound. This information is given with each package insert.

2. Use of a polycation is essential to overcome the surface negative charge on the cell and to achieve efficient infection with retroviral vectors. If polybrene at a concentration of 4–8 μg/mL is unacceptably toxic to a given cell line, then substitution of a different polycation can be tried. Protamine sulfate (4–8 μg/mL) or poly-L-lysine (5μg/mL) are also effective.
3. With one round of centrifugation, vector stocks can easily be concentrated 100-fold with >90% recovery of infectious particles (Burns *et al.*, 1993). If further concentration of vector is required, a second round of centrifugation achieves an additional 10-fold concentration of the vector with approximately 75% recovery of infectious particles.
4. If a different retroviral genome is constructed that expresses a protein that can be readily assayed, then colonies of the producer cell lines can be individually selected and screened for protein expression. In this way, one can select a clone with high transcriptional activity that will yield the highest titre of virus. The variability in protein expression between clones is attributed to position effects that vary with the integration site of the provirus.
5. In addition to the virus particles with their RNA genome, culture supernatants also contain contaminating proteins as well as DNA fragments from the producer cells containing provirus. Therefore, cells must be washed extensively to remove contaminating provirus derived from the producer cells before DNA is extracted for PCR analysis to confirm infection. Alternatively, a method of DNA extraction in which nuclei are separated from membranes before lysis may be used.
6. A pair of PCR positive control primers should always be used when testing DNA extracted from cell lines. Many published primers are available that will amplify the genomes of various insect species. Primers for the actin genes frequently cross-hybridize between species (Matsubara *et al.*, 1996) and may be tried. Uninfected insect cell line DNA and a tube containing no DNA template should also be included as negative controls for the LTR PCR assay.

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CHAPTER THIRTY EIGHT

Viral expression systems and viral infections in insects

Stephen Higgs, Ken E. Olson, Kurt I. Kamrud, Ann M. Powers and Barry J. Beaty

38.1 INTRODUCTION

The development of techniques to manipulate reliably the arthropod genome in a predictable manner remains an elusive goal. To date, all successful transformations of mosquitoes have been due to illegitimate recombination, and transformation efficiencies are too low for routine use. The routine generation of transgenic mosquitoes will undoubtedly be achieved, but when transformation technology is finally perfected, it would be beneficial to have a battery of sequences of known utility (e.g. disruption of pathogen transmission) for ready production of mosquitoes with the desired characteristic. In the absence of heterologous gene expression from within the mosquito genome, virus expression systems can be exploited to introduce and express sequences in mosquito cells for functional analysis. The Sindbis (SIN) alphavirus expression systems have proven to be exceptionally useful for expression of heterologous genes and for gene knock out. Additionally, viral vectors such as *Aedes aegypti* densonucleosis parvovirus and pseudotyped retroviral expression vectors (Carlson *et al.*, 1995; Chapter 37) may facilitate mosquito transformation.

Infectious, full-length cDNA clones have been developed for several mosquito-borne alphaviruses (Davis *et al.*, 1989; Kuhn *et al.*, 1991; Liljestrom *et al.*, 1991; Rice *et al.*, 1987), and expression systems have been developed from some of these (Rice *et al.*, 1987; Xiong *et al.*, 1989; Geigenmuller-Gnirke *et al.*, 1991; Liljestrom and Garoff, 1991; Bredenbeek and Rice, 1992; Bredenbeek *et al.*, 1993). The biology and molecular genetics of alphaviruses have been well described (Strauss and Strauss, 1994) and are not detailed here. Rather, we describe the expression systems based upon the SIN virus, since this system has proven utility for expressing heterologous gene sequences in a variety of mosquito species. SIN is an *Alphavirus* that is typically transmitted by *Culex spp.* and infects a wide range of vertebrates including man. To put the usefulness of this expression system in context, it is necessary to describe briefly the systems from which it was derived, and to speculate on future developments. In addition to describing specific methods for research with SIN expression vectors, we also describe some general methods for studying other arboviruses, since these are not covered elsewhere in this manual.

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38.1.1 pTRCAT

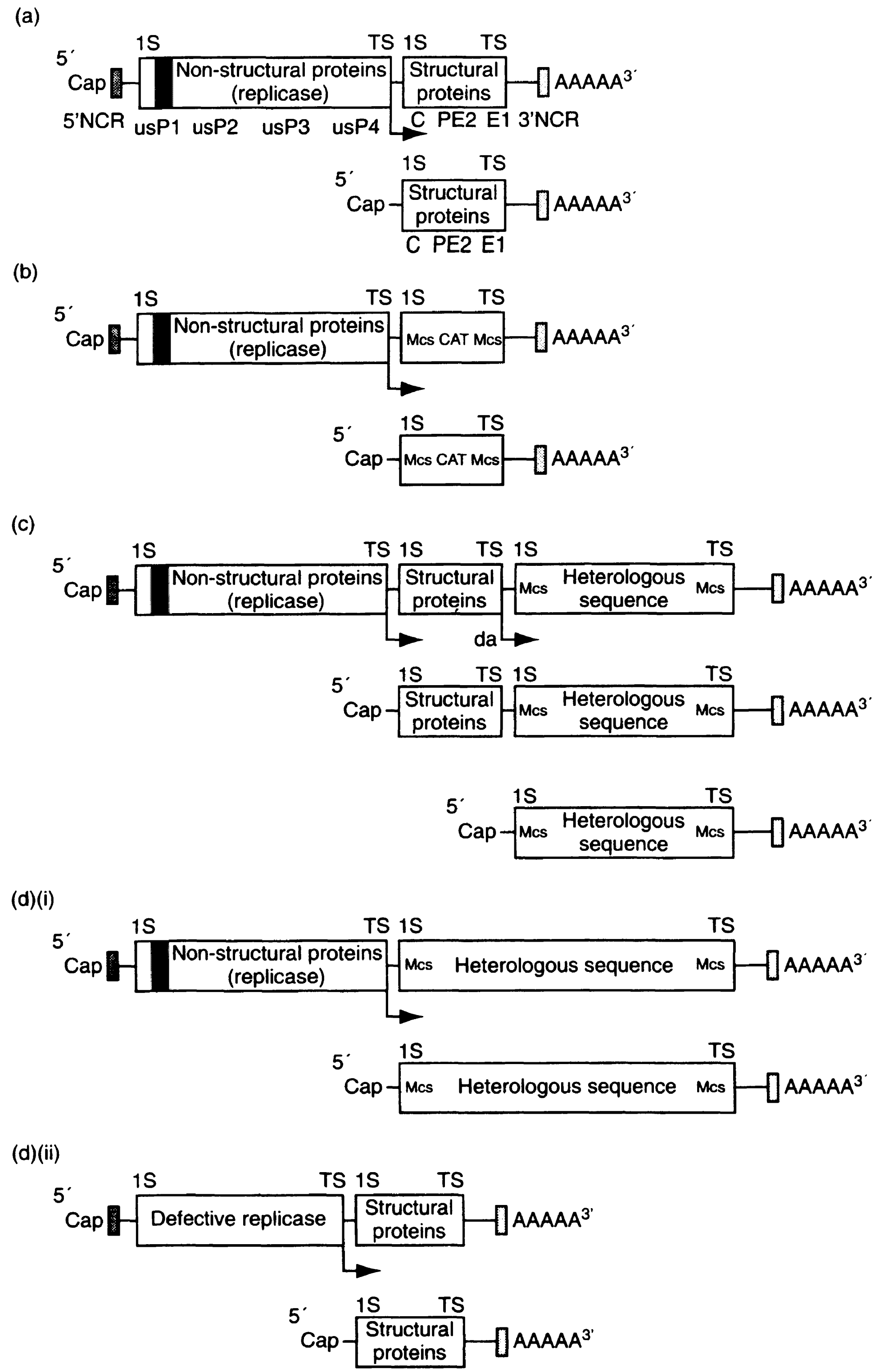
An early *Alphavirus* expression plasmid termed pTRCAT (Figure 38.1b) contained cDNA from a defective interfering (DI) RNA genome of SIN virus (Xiong *et al.*, 1989). Low transfection efficiencies, particularly in *Ae. albopictus* mosquito (C6/36) cells, have limited the usefulness of these first generation SIN expression plasmids for mosquito research (Olson *et al.*, 1992). However, since TRCAT is essentially a replicon, the system is considered further in Section 38.1.3.

38.1.2 Properties of the double subgenomic sindbis virus expression system

A significant development in alphavirus expression technology was the production of an infectious, recombinant dsSIN expression system (Hahn *et al.*, 1992). The dsSIN expression system contains a full-length SIN viral cDNA with a second internal subgenomic RNA promoter positioned either upstream or downstream of the internal RNA promoter and coding region for the SIN structural proteins (Figure 38.1c). Immediately downstream of the second promoter heterologous cDNA sequences such as CAT can be inserted. An important aspect of the dsSIN expression system is that the genomic RNA transcribed, *in vitro*, from the dsSIN plasmid template is competent for both viral replication and genome packaging. When the RNA is transfected into permissive cells, infectious, recombinant virions are produced that can efficiently deliver the dsSIN genomic RNA to animal cells.

Cells transfected with dsSIN genomic RNA transcribe three RNA species: the genomic dsSIN RNA, the subgenomic mRNA encoding the viral structural proteins and the subgenomic mRNA encoding the heterologous sequences (Figure 38.1). High titres ($>9.0 \log_{10} TCID_{50}/mL$) of recombinant dsSIN virus can be generated from transfected BHK-21 cells, which can then be used to infect other cells to obtain highly efficient expression of a heterologous gene. CAT and various biologically important genes have been successfully expressed in recombinant dsSIN viral infected vertebrate and mosquito cells (Hahn *et al.*, 1992; Olson *et al.*, 1993; Piper *et al.*, 1992; Powers *et al.*, 1994), and in mosquitoes (Carlson *et al.*, 1995; Higgs *et al.*, 1993, 1994; Olson *et al.*, 1993; Rayms-Keller *et al.*, 1995). The protocol for producing infectious virus from the dsSIN plasmids is described below (summarized in Figure 38.2). SIN virus infections in mosquito cells are persistent and noncytopathic. In contrast, the dsSIN viruses, like wild-type SIN virus, produce a lytic infection in permissive vertebrate cells.

Figure 38.1 *Alphavirus*-derived expression vectors. Translated regions of the genomic and subgenomic RNAs are shown as open boxes. The genes encoding the nonstructural proteins (nsP1, nsP2, nsP3, nsP4), structural proteins (C, PE2, E1) and heterologous protein (if any) are not to scale. *Cis*-acting sequences important for replication and transcription [the 49 base 5' noncoding (5'NCR) and the 3' noncoding region (3'NCR)] are shown as hatched boxes, and the sequence in the nonstructural region (nsP1) important for encapsidation is shown as a solid box. Subgenomic mRNAs encoding the encapsidated proteins are shown below each RNA. Translation initiation (AUG) and termination signals are denoted as IS and TS, respectively. Arrows show the start sites for subgenomic mRNA transcription, i.e. the subgenomic mRNA promoter (an internal initiation site), on the negative-strand genome-length template. (A) Structure of the parent *Alphavirus* genome RNA; (B) structure of TRCAT showing the CAT gene inserted between multiple cloning sites (Mcs); (C) infectious *Alphavirus* vector (TE/3'2J) RNA containing a duplicated site (ds) for subgenomic mRNA transcription to express the heterologous gene; (D) replicon system showing (i) the packaged plasmid, which lacks the structural genes, and (ii) the helper plasmid that provides structural genes but lacks the *cis*-acting sequences and so cannot itself be packaged. (Reprinted with permission from Higgs *et al.*, 1993.)


(a)
5′
Cap
1S
TS
Non-structural proteins
(replicase)
1S
TS
Structural
proteins
AAAAA3′
5′NCR
usP1
usP2
usP3
usP4
C
PE2
E1
3′NCR
(b)
Mcs CAT Mcs
(c)
Mcs
Heterologous
sequence
da
(d)(i)
Heterologous sequence
(d)(ii)
Defective replicase

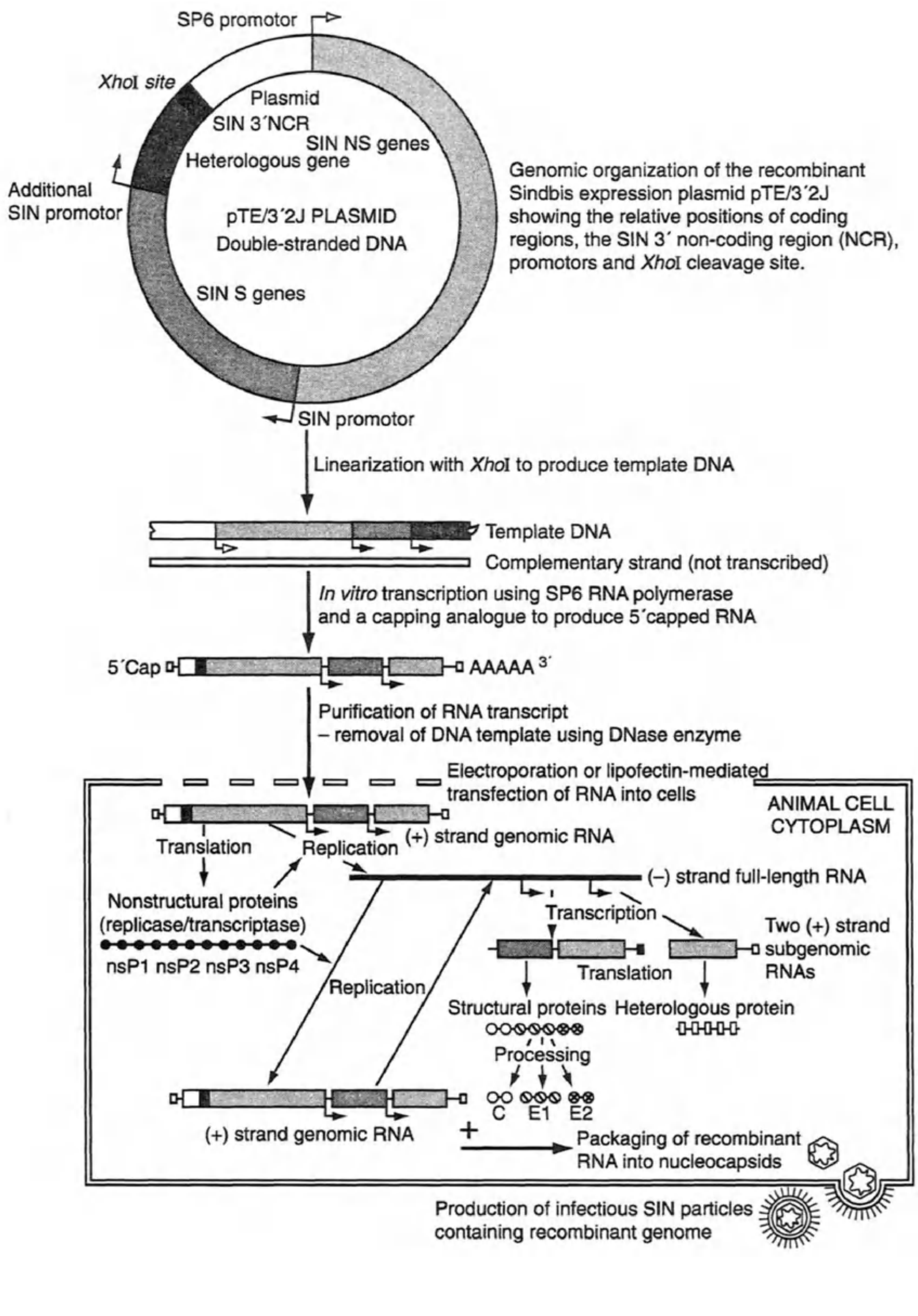


Figure 38.2 Outline of the protocol for producing infectious virus from double-subgenomic SIN expression plasmids. (Reprinted with permission from Higgs *et al.*, 1993.)

The dsSIN recombinant viruses have a unique combination of features that make them particularly valuable as expression vectors in mosquitoes: (i) recombinant virus can be generated to high titre within 30 h post-transfection; (ii) virtually 100% of the cultured cells can express the heterologous gene after infection with the recombinant virus at a high multiplicity of infection (moi); (iii) the genome functions as a mRNA and encodes its own replicase for efficient replication and transcription; (iv) recombinant virus replicates in the cell cytoplasm, which obviates problems associated with 'nuclear' DNA expression systems (e.g. mRNA splicing, mRNA transport, and poorly characterized DNA promoters in mosquitoes); (v) the expression vector allows transcription and translation of the exogenous gene sequences in the cytoplasm with high efficiency; (vi) the expression vector persistently infects mosquito cells or mosquitoes producing no apparent cytopathic effects and provides long-term expression of a given gene; (vii) recombinant viruses, like wild-type SIN viruses, have broad host ranges, and under laboratory conditions, can probably infect a variety of insect species; (viii) the infectious viral expression vectors generate recombinant virus that can be administered to mosquitoes either by intrathoracic inoculation or by bloodfeeding (when applicable) to express proteins of interest.

Despite these features, certain characteristics of the dsSIN system do limit its usefulness. The dsSIN viral replication machinery utilizes an RNA-dependent RNA polymerase that, unlike DNA polymerases, has no editing function. Consequently, the genome of the recombinant viruses accumulates mutations. Another problem results from the packaging constraints of the icosohedral nucleocapsid, which can only encapsidate a limited quantity of RNA. If the recombinant genome is larger than the wild type genome, then deletion mutants are therefore more efficiently packaged, generating viruses that have lost the insertion. The combination of a large insert and increasing numbers of replication cycles greatly increases the probability of mutation. In practical terms, this means that the recombinant dsSIN virus generated immediately following cell transfection, i.e. first passage, should be used to infect cells for expression. Ideally, the heterologous sequence should be less than 1 kb, although, as discussed in Section 38.1.3, alternative expression systems may be utilized to extend this sequence size.

38.1.3 Properties of the alphavirus replicon systems

Whilst dsSIN viruses have been produced that contain heterologous genes as large as 3.4 kb, dsSIN viruses with inserts of <2 kb (preferably <1 kb) are more stable and tend to produce fewer deletion mutants (Bredenbeek and Rice, 1992; C.M. Rice, pers comm.). To increase the packaging capability and hence utility of an *Alphavirus* replicon, a suicide replicon expression system has been developed from SFV and SIN viral infectious clones (Liljestrom and Garoff, 1991; C.M. Rice, pers. comm.). The term suicide is used because the virus is capable of only a single round of infection since only the DI replicon RNA is packaged. Although the replicon RNA establishes persistent infections, the extent of the infection is limited to those cells initially exposed to virus. This limited replication potential has important biosafety considerations (Section 38.1.5). If recombination events occur during RNA replication after transfection, low levels of infectious virus will be produced.

The *Alphavirus* replicon system (Figure 38.1d) was originally developed for SFV (Liljestrom and Garoff, 1991). A similar system based on SIN has been used in mos-

quito studies (Kamrud *et al.*, 1995). RNA is transcribed *in vitro* from a SIN replicon plasmid (e.g. pSINrep5/CAT) and from a SIN DI virus helper construct (pDH-EB or pDH(26S)5′SIN). The replicon genome does not encode SIN structural genes, and so sequences of 4–5 kb can be inserted for expression without exceeding the SIN genome packaging constraints. Helpers encode viral structural genes and contain a viral packaging signal. Replicon and helper RNAs are co-electroporated into BHK-21 cells and are packaged individually or co-packaged, generating recombinant replicon viruses designated e.g. rep5/CAT/EB and rep5/CAT/26S (Kamrud *et al.*, 1995).

38.1.4 Unique restriction sites for cloning sequences into dsSIN vectors

A number of unique restriction enzyme sites, which facilitate cloning of heterologous genes of interest, are located immediately downstream of the second SIN subgenomic promoter. Since there are several vector variants, it is not practical to list all of these sites. From a practical standpoint, the *Xba*I has been the most useful in our work. Although an *Apa*I site is present, for most dsSIN viruses it cannot be used since this will disrupt virus production. Expression vectors, and the restriction maps for these, may be obtained from Dr C.M. Rice (USA) and Dr P.J. Bredenbeek (Europe), whose addresses are given in the Acknowledgements' section.

38.1.5 Safety

An aspect of research with all of these expression systems that cannot be overemphasized is safety. Arboviruses are classified according to various criteria and it is essential that researchers are aware of these (Beaty *et al.*, 1996). SIN is a human pathogen that can be grown to high titre ($>10^8$ infectious particles/mL) in the laboratory. In both the United States and United Kingdom, SIN is classified as a risk group 2 category pathogen. In other countries, SIN and recombinants of SIN may be classified differently; indeed certain uses may also change virus categorization. The consequences of categorization must be realized (Higgs and Beaty, 1996). All research must be conducted under appropriate levels of containment. These are described in Government literature (e.g. USDHHS, 1993; HMSO, 1990). Institutes where research is to be conducted typically have committees (e.g. Biosafety) that set out their own regulations. They must be consulted, and must provide approval for the planned research before the expression vectors are obtained. Each genetic manipulation of the expression vector may require new approval.

38.2 MATERIALS

1. 1× PCR buffer: 1.5 U of *Taq* polymerase (Promega Corp, Madison, WI), 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl pH 9.0, 0.1% Triton X-100, 200 μM of each deoxynucleotide triphosphate, 50 pM of each forward and reverse primer.
2. TE Buffer: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.
3. Proteinase K buffer: 0.5 M NaCl, 0.5 M EDTA, 0.1 M Tris-HCl, pH 8.0.

4. Transcription mix: 40 mM Tris-HCl (pH 7.6), 6 mM $MgCl_2$; 5 mM DTT, 2 mM spermidine, 1000 U/mL of human placental RNase inhibitor, 1 mM each ATP, CTP, UTP, and GTP, 1 mM capping analog [$m^7G(5')ppp(5')G$; Ambion], 100 μg/mL BSA, 400 U/mL SP6 polymerase (20 U/μg of DNA template, Rice *et al.*, 1987).
5. TSE Buffer: 0.1 M Tris-HCl pH 7.6, 0.15 M NaCl, 0.003 M EDTA pH 8.0.
6. 20×SSC Buffer stock (1×SSC = 150 mM NaCl, 15 mM sodium citrate): Dissolve 175.3 g of NaCl, and 88.2 g sodium citrate in 800 mL of water. Adjust pH to 7.4 with 10 M NaOH. Add water to final volume to 1 L, dispense in aliquots and sterilize by autoclaving.
7. Denhardt's solution: 3×SSC buffer, 0.02% Bovine serum albumin (fraction V), 0.2% Polyvinylpyrrolidone, 0.2% Ficol. Filter sterilize and store aliquots at –20°C.
8. Acetylation solution: 0.1 M triethanolamine, pH 8.0. Add acetic anhydride to 0.15% (w/v) – mix vigorously 10 min.
9. DABCO mountant: 9 parts glycerol to 1 part phosphate buffered saline (PBS), adjusted to pH 8.6 with NaOH. To prevent fading, add 1.4 Diazobicyclo (2,2,2) Octane (DABCO, Sigma # D-2522). Add 2.5 g DABCO to 90 mL glycerol, heat in a water bath until dissolved, add 10 mL PBS and adjust pH to 8.6.
10. Fixative for autoradiographic detection of virus: 4% paraformaldehyde, 0.8% gluteraldehyde, 0.1 M sodium cacodylate pH 7.3.
11. ELISA diluent: 0.01 M PBS, pH 7.5, 10% heat-inactivated fetal bovine serum, 250 U/mL penicillin, 250 μg/mL streptomycin and 2.5 μg/mL amphotericin B.
12. ELISA wash buffer: PBS pH 7.2 containing 0.1% Tween 20.
13. RNA extraction buffer (solution D): 4 M guanidine thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sodium sarkosyl, 0.1 M β-mercaptoethanol.
14. Solubilization buffer: 5 M guanidine isothiocyanate, 100 mM EDTA, pH 7.0, 10 mM dithiothreitol.
15. Denaturing solution: 7% formaldehyde, 6×SSC.
16. Prehybridization solution: 50% formamide, 6×SSC, 5× Denhardt's solution, 0.1% SDS, 200 μg/mL salmon sperm DNA.
17. RNA Probe: prehybridization solution, α^{32}P-UTP labelled RNA (5×10^5 cpm/cm^2 of filter).
18. RNase A solution: 20 μg/mL RNase A, 20 U/mL RNase T1, 50 μg/mL poly A, 2×SSC, 100 mM EDTA.
19. *In situ* hybridization mix: 1.67 pg/μL suitably labelled (e.g. biotin or ^{35}S) probe RNA, 50% formamide, 2×SSC (see above), 0.2% BSA, 10% dextran sulfate, 100 mM DTT, 500 ng/μL tRNA.
20. 1× first strand buffer: 10 mM DTT, 10 μM dNTPs, 40 U/μL RNasin.
21. Fixative-stain for titrations: amido black 0.1%, acetic acid, 10%, isopropanol 25%.

38.3 METHODS

38.3.1 Construction of recombinant viruses

To generate recombinant SIN expression viruses, we routinely insert heterologous gene sequences into the *Xba*I site of either the TE/3'2J (dsSIN) or SINrep5 (replicon) plasmids. If no convenient restriction sites are available in the sequence of interest for cloning into this site, the following method is used.

We have found that the easiest method of cloning the heterologous sequence is by amplifying the sequence by PCR for DNA/cDNA sequences and by RT-PCR for RNA sequences. If necessary, oligonucleotide primers flanking the heterologous sequence are synthesized with an in frame ATG start codon at the 5′ end of the forward primer and an in frame TGA stop codon at the 5′ end of the reverse primer.

All PCRs are performed by adding 1 ng of template DNA to 50 μL of PCR buffer. The mixture is thermocycled at 94°C for 1 min, 55°C for 1 min and 72°C for 2 min through 25 cycles with a final extension time of 7 min at 72°C. If working from an mRNA sequence, the reverse primer is initially used to synthesize cDNA in the presence of reverse transcriptase before continuing with PCR (Section 38.3.8c).

The amplified heterologous sequence is TA cloned (Marchuk *et al.*, 1990) into a modified Bluescript II SK+ plasmid (Stratagene Inc. La Jolla, CA) that contains an *Nhe*I site in the polylinker. The modified Bluescript plasmid can easily be generated by digesting the plasmid with *Hin*dIII restriction endonuclease and filling in the 5′ overhangs with the Klenow fragment of DNA polymerase I. The plasmid is religated, creating an *Nhe*I site which has compatible ends with *Xba*I. TA cloning of this modified plasmid is accomplished by digesting the plasmid with *Eco*RV and adding dTTP to the 3′ ends of the plasmid. TA cloning follows the procedure described by Marchuk *et al.* (1990) and takes advantage of the ability of *Taq* polymerase to add dATP to the 3′ ends of PCR products. We routinely clone PCR products into the modified plasmid as follows:

1. 50 μg of the modified Bluescript plasmid is digested with *Eco*RV. The digested plasmid is then purified by phenol/chloroform extraction or column purifications (Wizard prep. columns, Promega, Madison, WI).
2. Approximately 10 μg of the digested plasmid DNA is incubated with 2.0 μL of 10× *Taq* polymerase buffer (2.5 mM $MgCl_2$; Promega), 4.0 μL of 10 mM dTTP and 15 U of *Taq* polymerase (Promega) for 2 h at 70°C. The plasmid is purified as described above.
3. The amplified heterologous sequence can now be ligated into the TA-ready plasmid using standard cloning techniques.

The heterologous sequence can now be excised from this plasmid with *Nhe*I and *Xba*I restriction endonucleases, and inserted at the *Xba*I site of pTE/3′2J. Recombinant clones are screened by PCR for orientation of the insert using one primer derived from pTE/3′2J and another primer derived from the insert sequence. The insert orientation is also confirmed by sequence or restriction analysis.

38.3.2 Production of recombinant virus

To produce infectious virus two steps are necessary (Figure 38.2). First, plasmid DNA must be linearized to allow *in vitro* run-off transcription of the RNA genome from the SP6 promoter. Second, RNA must be introduced into cells.

(a) Linearization

pTE/3′2J template DNA is linearized with *Xho*I. To ensure complete digestion of the plasmid we routinely use 3–4 U of *Xho*I for each μg of DNA.

1. For a 500 μL digestion volume, pipette the following volumes into a sterile 1.7 mL capacity microcentrifuge tube (note that the enzyme should be added last):

Water 37 μL, 50 μL 10× H Buffer (Boehringer Mannheim, Indianapolis, IN, or suitable alternative) 50 μL, BSA (1 mg/mL) 50 μL, 100 μg of DNA (based on a concentration of 300 μg/mL = 333 μL), 30 μL (i.e. 300 U) of *Xho*I enzyme (at 10 U/μL).

2. Incubate in a water bath, 37°C, 3 h.
3. Run 5 μL of the digestion mix on a 1% agarose gel. In different lanes, run a DNA ladder, the digested DNA and an undigested sample against which to compare. If completely digested, the linearized DNA will run slightly higher than the undigested. An incomplete digestion will be indicated by the presence of two bands: one with the same mobility as seen for the undigested DNA and one higher. If completely digested, proceed with following steps. If incomplete, continue with incubation and recheck. DNA must be free of enzyme and salts etc., and so is extracted from the digestion mix.
4. For the 500μL digestion volume, add 1 volume (500 μL) of Tris-saturated Phenol, pH 8.0.
5. Vortex, then centrifuge, 14 000 rpm, 5 min.
6. Transfer aqueous (upper) layer to new tube.
7. Add 1 vol (500 μL) chloroform/isoamyl alcohol (24:1).
8. Mix, then centrifuge, 14 000 rpm, 2 min.
9. Transfer aqueous (upper) layer to new tube.
10. Add 0.1 vol (i.e. 50 μL) of 3 M sodium acetate and 2 vol (1 mL) of ethanol.
11. Precipitate DNA at –20°C, 1 h.
12. Centrifuge, 14 000 rpm, 4°C, 20 min.
13. Carefully remove supernatant without disturbing the fine DNA pellet.
14. Carefully add 70% ethanol.
15. Centrifuge, 14 000 rpm, 4°C, 10 min.
16. Carefully remove supernatant without disturbing washed DNA pellet.
17. Dry pellet by evaporating any remaining supernatant at 37°C.
18. Add 0.1 vol (i.e. 50 μL) of 10× proteinase K buffer.
19. Add 0.1 vol of 5% SDS.
20. Add self-digested proteinase K to a final concentration of 100 μg/mL.
21. Incubate, 37°C, 1 h.
22. Repeat steps 4–17.
23. Resuspend dried pellet in DEPC (ie RNase-free) treated water to 1 μg/μL.

(b) In vitro *transcription*

1. In a clean 1.5 mL microfuge tube, add e.g. 50 μg/mL linearized DNA template to the transcription mix.
2. Incubate, 39°C, 1 h.
3. Determine the integrity of transcript by electrophoresis in a formaldehyde denaturing agarose gel before storing the RNA at –70°C. We routinely transfect RNA immediately after *in vitro* transcription without further processing.

(c) Production of infectious virus

To produce recombinant dsSIN or replicon viruses, we electroporate the positive sense genomic RNA into BHK-21 cells. If researchers choose to use other lines, conditions should be carefully optimized. Although we used lipofectin to introduce RNA into cells, we have found electroporation to be more efficient and consistent. Other alternatives are probably also suitable.

(d) Electroporation

RNA may be electroporated into BHK-21 cells without further purification. We use an ElectroCell Manipulator (BTX Inc., San Diego, CA).

1. Grow BHK-21 cells to 70–80% confluency.
2. Trypsinize cells and transfer to centrifuge tube.
3. Centrifuge at 1500 rpm, 5 min.
4. Discard supernatant and resuspend cells in cold PBS pH 7.4.
5. Wash three more times by repeating steps 3–4 and finally resuspend cells in DEPC-treated PBS to give 10^7 cells/mL.
6. Transfer 0.4 mL of cells to a 2 mm gap cuvette (BTX Inc.).
7. Add RNA (typically at 1μg/μL, total of 10 μg for dsSIN, or 5 μg replicon plus 5 μg helper).
8. Place cuvette in manipulator and pulse twice in succession at RT, 450 V, 100 μF, 720 ohms (pulse lengths ranging between 0.7–0.9 ms).
9. Immediately after pulsing, add 0.6 mL L-15 medium, 10% FBS to the electroporated cells.
10. Transfer cells to a 25 cm^2 flask containing 9.0 mL L-15 medium, 10% FBS.
11. Incubate, 37°C until >70% of the cells showed CPE (typically 24 h or 36–48 h post-electroporation for dsSIN and replicon respectively).
12. Transfer L-15 medium to centrifuge tube.
13. Clarify by centrifugation at 1000 g, RT, 3 min.
14. Store virus at –70°C.

38.3.3 Virus concentration

Virus stocks of dsSIN constructs may be as high as 10^9 pfu/mL, but replicon viruses are frequently lower. Since it is preferable to use as high a titre as possible to infect mosquitoes, we often increase the titre by concentration. Virus can be amplified by passage in BHK-21 cells before concentration. Rep5/CAT/EB and rep5/CAT/26S viruses were concentrated using a modified version of the procedure described by Obijeski *et al.* (1976).

1. Load virus stock onto a cushion of 30% (w/v) sucrose in TSE buffer.
2. Centrifugation at 20 000 rpm, 3 h, 4°C.
3. Discard supernatant.
4. Resuspend virus pellet in TSE containing 20% FBS as a virus stabilizer.
5. Store at –70°C.

38.3.4 Transcript characterization

Transcripts may be characterized to check for the presence of the insert as follows:

1. Infect e.g. C6/36 cells in suspension at a moi of 10 in 1 mL Leibovitz L-15 medium.
2. Transfer cells to 25 cm^2 flasks and incubate at 28°C.
3. At 24 h post-infection, wash monolayers twice with PBS.
4. Isolate total cellular RNA by guanidinium thiocyanate extraction (Chomczynski and Sacchi, 1987).

5. Separate RNA electrophoretically on a formaldehyde denaturing gel (Sambrook *et al.*, 1989).
6. Transfer RNA to a charged nylon membrane (Boehringer Mannheim, Indianapolis, IN).
7. Hybridize in 50% formamide, 6×SSC, 5× Denhardt's solution, 0.1% SDS, and 200 μg/mL salmon sperm DNA for 12 h using 3×10^5 cpm/mL ^{32}P-labelled specific probe (e.g. plasmid or insert).
8. Expose the filter to X-ray film until a suitable image is produced (e.g. for 72 h).

38.3.5 Virus quantitation

Since dsSIN viruses cause cytopathic effect (CPE) in vertebrate cells, they are easily quantified by standard titration techniques (Section 38.3.9). Replicon viruses are more difficult to quantify because they do not replicate and disseminate from the cells that they initially infect. Kamrud *et al.* (1995) have described an immunofluorescent focus assay. Basically, dilutions of virus stock are allowed to adsorb onto a cell monolayer grown on glass coverslips (Gould *et al.*, 1985). At 6 h post-infection cells are fixed in cold acetone and stained using antibodies reactive with SIN proteins or with the heterologous protein of interest (Section 38.3.7b(i)). The number of fluorescing (i.e. infected) cells is used to calculate the virus titre.

38.3.6 Infecting mosquitoes with viruses

Mosquitoes may be infected with viruses in basically two ways: (i) orally (*per os*) e.g. using artificial membrane feeders, and (ii) parenterally, e.g. by intrathoracic inoculation. Whilst *per os* methods most closely approximate natural infections, they are rarely able to infect all mosquitoes. Although SIN orally infects several species of arthropod (Karabatsos, 1985), the relative efficiency varies considerable (Schiefer and Smith, 1974). The dsSIN expression viruses were produced from a virus selected for neurovirulence in vertebrates, which may explain the relatively low oral infectivity for mosquitoes. Work is in progress to generate more mosquito-tropic SIN expression vectors. Parenteral infections effectively provide a method by which most mosquitoes can be infected. This method circumvents the midgut infection barrier and so, although events in midgut epithelial cells cannot be monitored, subsequent events in other susceptible mosquito tissues seem to be no different from *per os* (i.e. natural) initiated infections.

(a) Per os *infections*

Mosquitoes can be orally infected by feeding on infected, vireamic vertebrates. To maximize infection rates, this method depends upon using the vertebrate when vireamias are at their peak. It is often difficult to measure precisely when this time is. With recombinant viruses, which have a tendency to lose inserts with increasing replication cycles, we have not used infected vertebrates. If this method is preferred over others, it is essential that vireamia patterns are determined and shown to be consistent. Appropriate approval for animal use must be obtained. An alternative method for oral presentation of virus to mosquitoes involves the use of artificial

meals and feeders. Virus is grown in cell culture (suckling mouse brain can be used), and the virus suspension is used to prepare a blood meal. We have used the following methods successfully.

(i) Blood

Blood should be as fresh as possible (we prefer <14 days old). Since different mosquito species may have particular host preferences, the blood source may influence feeding success. For *Ae. aegypti*, we collect sheep blood in 100 mL bottles containing 20 U Heparin (Sigma H-3393) per mL of blood. Bottles with Heparin in 5 mL sterile PBS are kept at 4°C until required. As blood is collected into the bottle (usually directly from an artery), it is swirled to ensure thorough mixing and to prevent clotting. Heparinized blood is stored at 4°C until required.

(ii) Washing the blood

Only erythrocytes (RBCs) are used to make up the artificial blood meal. Monocytes, cell debris resulting from haemolysis, and serum are discarded. Any antibodies that may react with viruses being used are therefore removed. Assume that for every volume of RBCs required (one third of total meal volume), at least twice as much whole blood should be washed just before meal preparation.

1. Gently centrifuge blood at 1500 rpm, 10 min. Do not use the brake since this often resuspends RBCs.
2. Carefully remove supernatant (including buffy coat if present).
3. Add PBS and gently mix by inverting the tube.
4. Repeat steps 1–3 until PBS remains clear (no free haemoglobin).

(iii) Artificial blood meal preparation

1. Mix together: 1 part washed RBCs, 1 part FBS containing 10% sucrose, 1 part virus suspension. To stimulate mosquitoes to engorge, adenosine 5′-triphosphate (ATP, Sigma A-6144) can be added to a final concentration of 0.02 M. Just before presenting the meal to mosquitoes, the meal should be heated to 37°C.

(iv) Meal presentation

Mosquitoes may have to be deprived of water and sugar before infection. Duration of deprivation should be established to minimize mortality. Times will depend on mosquito species, humidity of the insectary, etc. We remove sugar 24 h before the feed, and water 12 h prior to the feed. The complete blood meal can be pipetted as droplets onto the cage netting or soaked into absorbent cotton (pledgets), which are placed onto the netting. We prefer to use glass feeders (Rutledge *et al.*, 1964) with a jacket through which water at 37°C is circulated. A membrane is placed over the mouth of the feeder and the meal is pipetted into the chamber. Various designs, membranes and protocols are available for different vector types (Higgs and Beaty, 1996; Chapters 2 and 9).

To minimize loss of virus infectivity, meals should be presented for the minimum time necessary to feed the required number of mosquitoes. Feeding in a relatively cool room may allow mosquitoes to detect the warm meal quickly and so reduce feeding time. The disadvantage is that mosquitoes may become quiescent, and the

droplets/pledgets may cool and become unattractive. Breathing onto the cage usually stimulates mosquitoes to feed, since CO_2 is an attractant used by the mosquito for host location.

(b) Inoculation

Inoculation equipment is relatively inexpensive and readily available (Rosen and Gubler, 1974; see also Chapter 9). Pathogens are usually inoculated into the thorax using glass needles made from capillary (haematocrit) tubes. The tubes are drawn into a fine tip over a flame or prepared using special apparatus (e.g. Narishige PB-7 puller and EG–4 beveller). Divisions may be stamped onto the needles to provide a measure of the inoculum quantity. Needles are held in the glass holder supplied with the tubes and this is attached to a syringe with a three-way valve *via* plastic tubing. The valve allows easy control of the inoculation and reduces the risk of reflux into the syringe. Adult vectors are immobilized by chilling in a refrigerator or on ice. Larvae can be anaesthetized for inoculation by immersion in ice cold water, but should not be immersed for long since immobilization will prevent respiration. After inoculation they are transferred to cartons before recovery. A chill table can significantly increase inoculation efficiency, especially in warm environments.

38.3.7 Detection of virus and virus products

Once vectors have been infected, and after an appropriate extrinsic incubation period, the presence of pathogens must be confirmed. RNA and protein products expressed by dsSIN viruses may be identified using various techniques. Both qualitative and quantitative methods can be employed. Antigenic protein is most easily located using appropriate antibodies in immunodetection techniques. Viral RNA may be detected using molecular techniques, and infectious virus may be detected and quantified on the basis of virally induced pathogenesis in certain cell culture systems. We have not utilized all of the techniques available (e.g. Sections 38.3.7b(i) and (ii)), but describe selected ones which may be of interest to readers. For some techniques, tissues may be simply squashed onto ethanol-washed microscope slides, but since several of the methods rely upon firm attachment of tissues to microscope slides, two methods to maximize adherence are described below.

(i) Poly-d-lysine coating of slides

1. Boil slides in 0.1 M HCl, 5 min.
2. Rinse slides thoroughly in ddH_2O.
3. Dehydrate slides by immersion in ethanol.
4. Mix poly-d-lysine in ddH_2O. Store stock at –20°C. It can be used repeatedly.
5. Immerse slides in poly-d-lysine, 30–60 min.
6. Rinse slides thoroughly in two changes of ddH_2O.
7. Dry slides, 37°C, overnight.
8. Store slides at 4°C in a sealed container to exclude moisture.

(ii) Organosilanation of slides for attachment of tissues

1. Wash slides in 1 M HCl, RT, 20 min.

2. Rinse in ddH_2O.
3. Dehydrate slides in ethanol series, 50%, 70%, 95%, 100%, 5 min each.
4. Coat slides with Denhardt's solution, 65°C, overnight.
5. Fix slides in ethanol:acetic acid (3:1), RT, 20 min.
6. Air-dry.
7. Immerse slides in acetylation solution.
8. Wash twice in ddH_2O.
9. Wash 95% ethanol, RT, 5 min.
10. Air-dry and store at RT.
11. Organosilanation: Immerse in organosilane (gamma-aminopropyltriethoxy silane) 1%, pH 3.45 – adjusted with HCl), 70°C, overnight. Use plastic *not* metal racks etc.
12. Wash extensively in ddH_2O.
13. Bake in oven, 100°C, overnight.
14. Store slides if required, RT, in dust-free boxes, up to 6 months.

Before use:

15. Immerse slides in 10% gluteraldehyde (v/v) in PBS (without $CaCl_2$ and $MgCl_2$), pH 7.0, RT, 30 min.
16. Rinse in ddH_2O.
17. Stabilize slides by immersion in 0.1 M sodium metaperiodate, RT, 15 min.
18. Rinse in ddH_2O. Use slides within 14 days of stabilization (store in dust-free boxes).

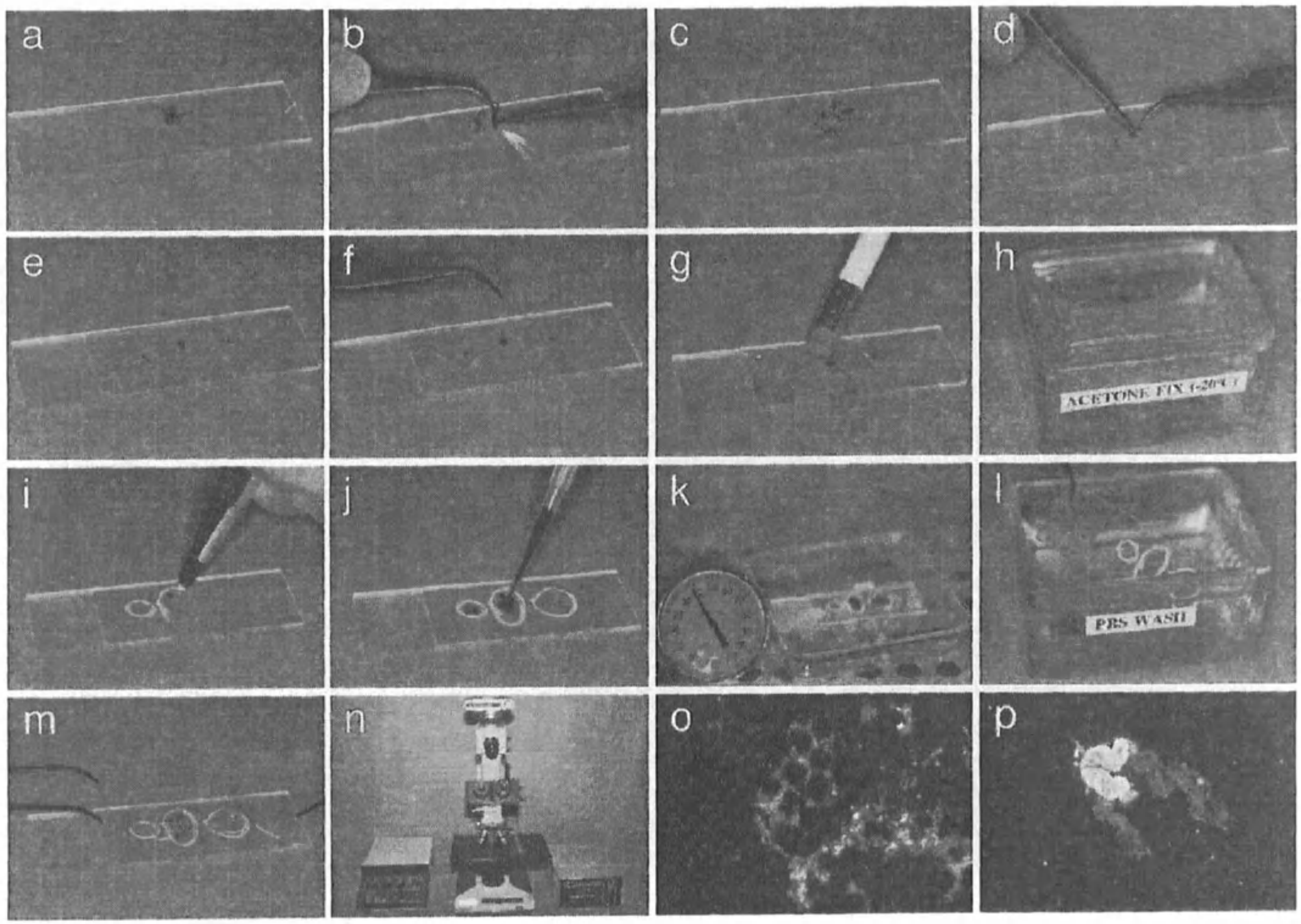
(b) Qualitative methods for virus detection

For qualitative analysis, immunodetection techniques are frequently used. These techniques all depend upon antibodies that react specifically with components associated with the pathogen. For viruses, these are most commonly the structural proteins, although modern molecular techniques, e.g. *in situ* hybridization, have now been developed to identify viral nucleic acids.

(i) Squash analysis for antigen

A typical direct immunofluorescence procedure is illustrated in Figure 38.3. When antigen is known to be abundant, antibodies that have been directly conjugated to a fluorochrome/enzyme may be used. Fluorescence eventually fades, and so as an

Figure 38.3. Detection of viral antigen in squashed mosquito preparations using a direct immunofluorescence technique. (A) A chilled/frozen mosquito is placed on an ethanol-washed microscope slide (do not freeze mosquitoes if intact organs, e.g. salivary glands are to be examined). Poly-d-lysine coated/organosilanated slides may be used to maximize adherence. (B)–(D) Legs and wings are removed, and the mosquito is dissected as required. Other parts are discarded. Single legs can be used to check mosquitoes for disseminated viral infections. (E) Parts (e.g. head thorax and abdomen) are arranged on the slide. (F) Ethanol-washed coverslips are placed over the tissues. (G) Tissue is squashed by pressing onto the coverslip (with e.g. an eraser) and dragging it across the slide. Organs (e.g. salivary glands) can be dried without squashing if preferred. (H) After air-drying, the tissue is fixed in cold acetone for 10 mins (30 min for whole organs). (I) After air-drying, tissues are separated by drawing around them with (e.g. a Tech-pen, Mark-Tex Corp). This also reduces the amount of reagent subsequently required. (J) Primary antibody conjugated to fluorescein is pipetted onto the tissue. (K) The slide is placed in a humidified chamber (e.g. Petri dish containing damp tissue) and incubated at 37°C for 40 mins. (L) The slide is rinsed in PBS. (M) Glycerol-saline mountant is pipetted onto the slide and tissue is covered with an ethanol-washed coverslip. (N) The tissue is examined using an epifluorescence microscope equipped with suitable filters. (O) Squash of thoracic tissue showing viral antigen (bright areas). In this typical example, the antigen is present in cytoplasm and not in the nuclei which are seen as black circles. (P) Whole salivary gland with antigen restricted to the proximal lateral lobes.


a
b
c
d
e
f
g
h
ACETONE FIX (-20°C)
i
j
k
l
PBS WASH
m
n
o
p

alternative to fluorescence detection, enzymatic detection can be used to produce permanent preparations (Section 38.3.7b(iii)). For precise location of antigen in vectors, both immunodetection techniques can be modified for use with organ mounts and for tissue sections. Both paraffin embedded and cryostat sections can be used, although temperature sensitivity of some antigens may limit the use of wax embedded preparation. Coloured substrates are available for different enzymatic reactions (e.g. Vector Laboratories, Burlingame, CA).

If a direct immunofluorescence procedure is not sufficiently sensitive to detect the antigen of interest, an indirect test may be used to amplify the signal. This is essentially as described in Figure 38.4 with the following modifications. The primary antibody is not conjugated. Between steps l and m (Figure 38.4) add the following steps:

1. Wash twice in PBS, pH 7.6.
2. Apply biotinylated secondary antibody [e.g. biotinylated sheep anti-mouse (e.g. Amersham Corp, Arlington Heights, IL)], 37°C, 40 min.
3. Wash twice in PBS.
4. Apply streptavidin–fluorescein (e.g. Amersham Corp.), 37°C, 10 min.
5. Wash in PBS.
6. Wash in ddH_2O.

(ii) Paraffin wax embedding of mosquitoes for sectioning
Modified from Faran *et al.* (1986) and Romoser *et al.* (1992).

1. Chill mosquitoes (do not freeze since this will destroy tissue integrity).
2. Remove wings and legs.
3. Inoculate formaldehyde (2–10% in PBS, pH 7.6) into thorax until solution exudes from coxae. Other fixatives may be suitable (Faran *et al.*, 1986).
4. Store in formal saline, RT, 2-20 h.
5. Transfer to 70% ethanol (store indefinitely).
6. Dehydrate in ethanol series, 70%, 90%, 100%, 100%, 100%, 60 min each.
7. Clear in Hemo-de (or other agent e.g. xylene), two changes, 30 min each.
8. Infiltrate: Hemo-de:paraplast (1:1) 55–60°C, 30 min; paraplast, 55–60°C, 60 min; vacuum oven, 380–500 torr, 55–60°C, 30 min. Embed in paraplast, 55–60°C, 60 min.
9. Cut 7–10 μm sections.
10. Mount sections on microslides (may be coated with Mayer's albumin).

(iii) Staining sections using avidin-biotin complex (ABC) – peroxidase method, based on Faran *et al.*, 1986 (see also Leake and Johnson, 1977). All steps at RT.

1. Deparaffinize: Hemo-de, 5 min. Hemo-de, 2 min.
2. Rehydrate in ethanol series, 100%, 95%, 70%, 2 min each.
3. ddH_2O, 2 min.
4. PBS pH 7.6, 3 changes, 5 min each.
5. Eliminate endogenous peroxidase activity by immersion in: 0.01 M periodic acid, 10 min; 0.01% sodium borohydrate, 10 min.
6. PBS, 3 changes, 5 min each.
7. Block potential secondary antibody sites with normal horse serum, 30 min.
8. Primary antibody, 60 min.
9. PBS wash, 3 changes, 5 min each.

10. Biotinylated secondary antibody (horse anti-mouse), 30 min.
11. PBS wash, 3 changes, 5 min each.
12. Avidin–biotinylated horseradish peroxidase complex, 30 min.
13. PBS wash, 3 changes, 5 min each.
14. Wash 0.05 M Tris-HCl pH 7.6, 3 changes, 5 min each.
15. Stain with diaminobenzidine (10 mg in 0.03% H_2O_2 diluted with Tris buffer), 6–8 min.
16. Tris wash (as step 14), 2 changes.
17. Counterstain in Mayer's hematoxylin, 1 min.
18. ddH_2O rinse and wash, 5 min.
19. Dehydrate in ethanol series, 70%, 95%, 95%, 100%, 2 min each.
20. Clear: Hemo-de, 5 min; Hemo-de, 2 min.
21. Apply coverslip with Permamount^R.

(iv) Staining sections using avidin–biotin complex (ABC) – fluorescein method, based on Higgs *et al.* (1993) and Olson *et al.* (1994).

1. Remove wax by immersion in xylene, two changes, 10 min each.
2. Rehydrate sections using ethanol series, 100%, 100%, 90%, 70%, 50% 10 min each.
3. Wash in PBS, pH 7.6, three changes, 5 min each.
4. Block potential secondary antibody binding sites with 1.5% normal serum from the species in which the secondary antibody was made, 37°C, 30 min.
5. Drain excess serum from slides.
6. Apply primary antibody at appropriate dilution, 37°C, 30 min.
7. Wash in PBS 3 changes, 5 min each.
8. Apply appropriate biotinylated secondary antibody 22 μg/mL, 37°C, 30 min.
9. Wash PBS, 3 changes, 5 min each.
10. Apply strepatavidin–fluorescein, 37°C, 10 min.
11. Wash PBS, 5 min.
12. Mount in DABCO mountant.

(v) Localization of virus in vectors by autoradiography, based on Weaver *et al.* (1991).

1. Prepare radiolabelled virus and purify using standard protocols.
2. Inoculate purified virus into host (e.g. chick jugular vein). Labelled virus could probably be successfully presented in an artificial meal.
3. Feed mosquitoes on host/meal for 60 min.
4. Chill and fix mosquitoes at desired time point. Remove legs and wings (and head perhaps), and immerse body in fixative, 24 h, 5°C. To allow fixative infiltration, puncture last abdominal segment and notum.
5. Separate thorax and abdomen to produce pieces that can be easily sectioned.
6. Dehydrate in acetone series, and embed in resin (e.g. Spurr's).
7. Cut 1 μm sections and collect on slides.
8. Dry, 60°C, 15 min.
9. Dip in autoradiographic emulsion (e.g. Ilford K.5D).
10. Dry coated sections, 23°C, 2 h.
11. Expose for suitable time (for ^3H labelled virus, maybe 3–4 months), 5°C.

12. Develop slides (e.g. Kodak D-19 developer).
13. Fix and apply coverglass prior to microscopic observation.

(vi) Localization of virus in vectors by electron microscopy, based on Sriurairatna and Bhamarapravati (1977); see also Weaver (1986).

1. Chill infected mosquitoes and fix in cold 2.5% gluteraldehyde.
2. Separate head thorax and abdomen to permit fixative infiltration and leave at 4°C, 4 h.
3. Wash pieces with cold PBS and store in PBS, RT until processed.
4. Post-fix in 1% osmium tetroxide, 2 h.
5. Embed in warm agar to allow easy handling.
6. Cut blocks into small cubes and dehydrate in ethanol series.
7. Infiltrate with propylene oxide.
8. Embed in resin (e.g. Epon 812).
9. Cut ultrathin sections and collect on grid.
10. Stain with uranyl acetate and lead citrate.
11. Identify virus particles by examining sections by transmission electron microscope.

(vii) Extraction of antigen from mosquitoes for enzyme-linked immunosorbent assays (ELISA)

1. Triturate mosquito pool (e.g. 100 individuals) with mortar and pestle in 2 mL ELISA diluent.
2. Centrifuge, 800 **g**, 4°C, 20 min.
3. Store supernatant at –70°C until needed for ELISA.

(viii) Mosquito ELISA

Based on Hildreth *et al.* (1982) and Wasieloski *et al.* (1994). Note that optimum concentrations of all ELISA reagents must be determined for each ELISA, typically by checkerboard titration.

1. Coat plate with capture antibody in 0.1 M carbonate-bicarbonate buffer, pH 9.25.
2. Incubate, 4°C, 18–24 h.
3. Wash plates 5× with ELISA washing buffer.
4. Incubate with 200 μL per well of 0.05 M *N*-acetyl cysteine, 37°C, 1 h.
5. Remove *N*-acetyl cysteine solution.
6. Add mosquito supernatants, 50 μL/well diluted 1:5 in 0.01 M PBS pH 7.5, 0.1% Tween20, 1.0% FBS.
7. Incubate, 37°C, 2 h.
8. Wash plates 5× with washing buffer.
9. Add e.g. rabbit detector antibody in 0.01 M PBS, 1% Tween20, 0.05% gelatin, 0.5% FBS, 1 mg/mL dextran sulfate.
10. Incubate, 37°C, 1 h.
11. Wash plates 5× with washing buffer.
12. Add 100 μL/well of biotinylated donkey anti-rabbit antibody (e.g. Amersham Corp.), diluted 1:1000 in 0.01 M PBS, 1% Tween20, and 1 mg/mL dextran sulfate.

13. Wash plates 5× with washing buffer.
14. Add 100 μL/well of streptavidin-peroxidase (e.g. Amersham Corp.) diluted 1:1000 in 0.01 M PBS, 0.1% Tween20, 1 mg/mL dextran sulfate.
15. Incubate, 37°C, 1 h.
16. Wash plates 5× with washing buffer.
17. Add 100 μL/well of suitable substrate (e.g. TMB).
18. When colour has suitably developed, stop by adding 100 μL/well of 1 M phosphoric acid.
19. Read absorbence at suitable wavelength (e.g. A_{450} for TMB).

38.3.8 Molecular methods of pathogen detection

Since molecular techniques are often highly specific and sensitive, the protocols described below have been designed and optimized to detect particular viruses. Probes and primers must obviously be designed for the pathogen/nucleic acid type of interest, and hybridization and PCR conditions (e.g. annealing temperatures and cycle details) must be optimized.

(a) Detection of viral RNA in mosquitoes by hybridization
Olson et al. (1990) and Ballinger et al. (1988) describe various molecular methods for pathogen detection. We describe mixed phase hybridization below, but consider liquid phase (solution) hybridization and in situ hybridization techniques to be too laborious for routine use with RT-PCR being the technique of choice.

(i) RNA extraction from mosquitoes for mixed phase hybridization, modified from Chomczynski and Sacchi (1987) (see also Chapter 15).

1. Triturate e.g. 25 mosquitoes in 2 mL solubilization buffer in Tissuemizer (Tekmar), high speed, 20 s.
2. Add: 0.1 vol (i.e. 200 μL) of 2 M sodium acetate, equal volume (2 mL) water saturated phenol, 0.2 vol (400 μL) chloroform. Vortex vigorously between each addition.
3. Vortex vigorously, 10 s.
4. Chill on ice, 10 min.
5. Centrifuge, 10 000 g, 4°C, 20 min.
6. Transfer aqueous phase (upper layer) to 15 mL, RNase-free siliconized Corex tube.
7. Add equal volume (2 mL) isopropanol, and allow RNA to precipitate at –20°C, 1–2 h.
8. Centrifuge 10 000 g, 4°C, 20 min.
9. Carefully remove supernatant, and briefly air-dry RNA pellet.
10. Resuspend pellet in 50 μL 0.5% SDS and store at –20°C.

(b) RNA blotting and mixed phase hybridization (Chapter 20)

1. Denature mosquito RNA (10 μg) in denaturing solution at 60°C, 15 min.
2. Blot 100 μL sample onto nylon membrane using suitable vacuum slot–blot apparatus.

3. Wash membrane briefly in 6×SSC.
4. Crosslink the RNA to the filter by exposing to UV radiation (160 J/m^2), 3 min.
5. Incubate filter in prehybridization solution, 60°C, 3 h.
6. Hybridize using fresh prehybridization solution containing suitably radiolabelled probe (5 × 10^6 cpm), 42°C, 16 h (42°C for formamide buffers, 60°C for aqueous buffers).
7. Wash filter 4× in 2×SSC with 0.1% SDS, RT, 5 min each.
8. Wash filter 2× in 0.1×SSC with 0.1% SDS, 60°C, 15 min each.
9. Wash filter 1× in 2×SSC with 10 μg/mL RNase A, RT, 10 min.
10. Air-dry filter and expose to X-ray film.

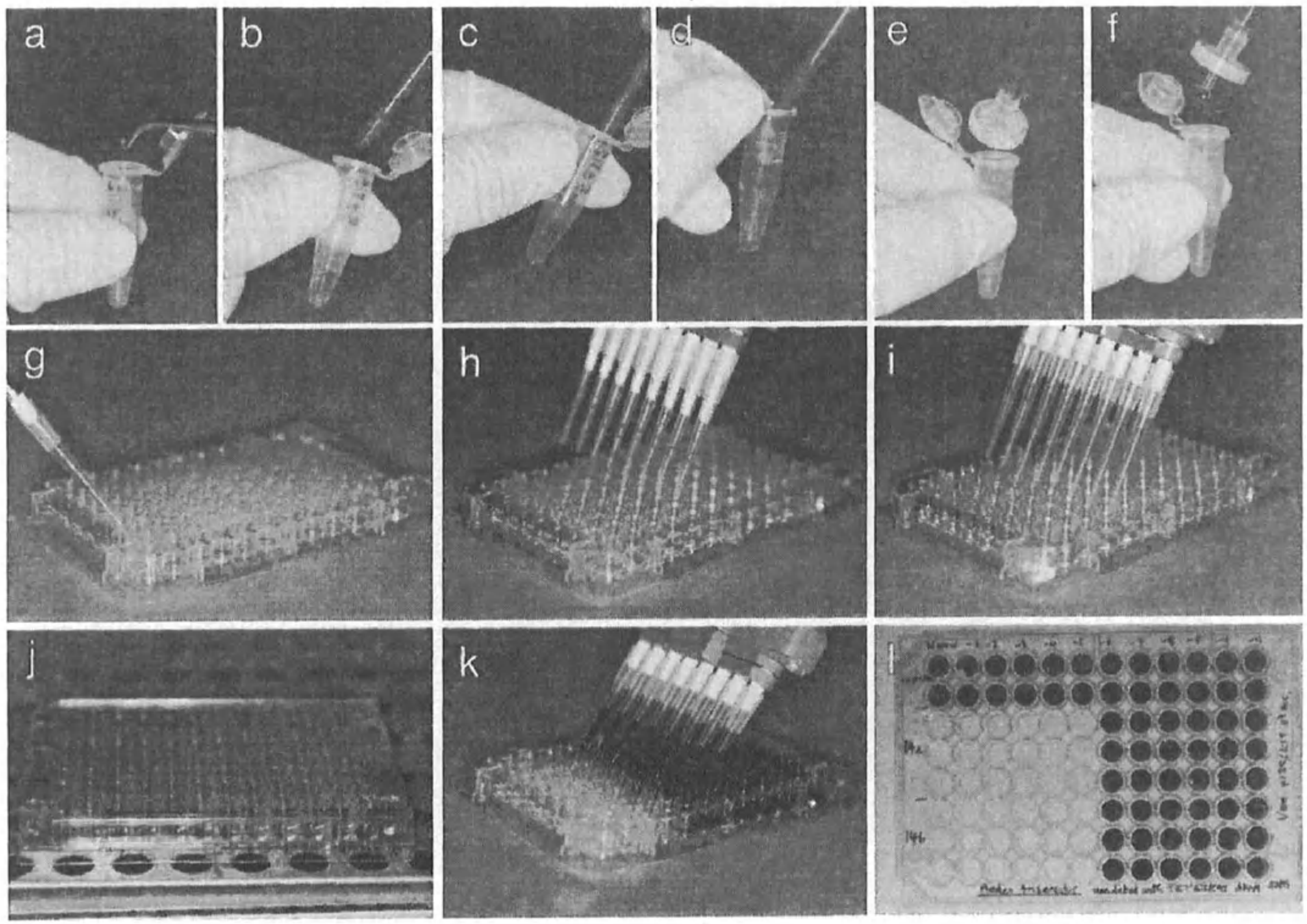
(c) RNA extraction from mosquitoes for reverse-transcription (RT)-PCR, from Wasieloski *et al.* (1994) (see also Chapter 15).

1. Triturate mosquito pools (e.g. 10 mosquitoes) in 500 μL of solution D. For pools of 50–100 mosquitoes, triturate in 2.0 mL of solution D, and increase other reagents accordingly.
2. Sequentially add and mix: 50 μL of 2 M sodium acetate pH 4.5, 500 μL of water-saturated phenol, 100 μL of chloroform/isoamyl alcohol (49:1)
3. Incubate on ice, 15 min.
4. Centrifuge, 10 000 **g**, 4°C, 20 min.
5. Save the aqueous phase and add an equal vol of isopropanol to precipitate the RNA.
6. Centrifuge, 10 000 **g**, 4°C, 20 min.
7. Carefully discard supernatant to leave RNA pellet.
8. Wash RNA pellet with 800 μL 3 M sodium acetate, pH 5.2.
9. Carefully discard supernatant to leave RNA pellet.
10. Wash RNA pellet with 70% ethanol.
11. Carefully discard supernatant to leave RNA pellet.
12. Dry at RT.
13. Resuspend pellet in 50 μL of DEPC-treated water.

(i) RT-PCR (Chapter 21).

1. Incubate one tenth of the total RNA (5 μL) from each sample at 70°C, 10 min with 15 pM of Primer-1 in a total volume of 10 μL. Primers are typically designed using computer programs and are not described here (Chapter 21).
2. Cool to RT.
3. Reverse-transcribe the RNA with 67 U SuperScript (BRL, Gaithersburg, MD) in 1× first strand buffer (20 μL reaction), 42°C, 1 h.

Figure 38.4 Extraction and quantitation of virus from mosquitoes. (A) An individual mosquito is placed in a labelled 1.7 mL microfuge tube. (B)–(C) 100 μL of diluent (e.g. medium for cell culture) is pipetted into the tube, and the mosquito is triturated using a suitable pestle until no recognizable parts remain. Tubes are placed on ice until all are processed. (D) Additional diluent (e.g. 900 μL) is added to the tube. (E)–(F) Mosquito triturate is loaded into a 1 mL syringe and is filtered through a sterile 0.22 μm pore-size filter and collected in a sterile 1.7 mL microfuge tube. (G) Samples (e.g. 100 μL) are pipetted into a sterile 96 well microtitre plate. (H) Replicate samples are titrated (e.g. in serial 10-fold dilutions) across the plate. (I) Suitable cells (e.g. Vero) in suspension are added to all wells. If susceptible cells are not available, plates can be stained and examined for fluorescence. (J) The titration plates are incubated at 37°C until cytopathic effect (CPE) is observed and complete. (K) Fixative-stain is added to all wells. (L) Wells with CPE are identified (clear wells), and virus titres are calculated [e.g. tissue culture infectious dose 50% ($TCID_{50}$)] using a suitable formula.



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4. Add 2 μL of cDNA to 48 μL of 1× PCR buffer, 1.5 U of *Taq* polymerase (Promega, Madison, WI).
5. Conduct PCR reaction in thermocycler under previously optimized conditions.
6. Analyse PCR products by electrophoresis in 1.5% agarose gels.

38.3.9 Quantitative methods of pathogen/antigen detection

If information on pathogen development in vectors is required, e.g. to determine growth dynamics of viruses in mosquitoes, samples collected at various times after infection can be quantitatively examined. For viruses, this assay is generally based on the detection of virus cytopathic effect in cell cultures challenged with sequential dilutions of virus. Cell culture methods for titration and plaque assay techniques are available in many microbiology texts and so are not described. Mosquito processing to isolate virus for titration is illustrated in Figure 38.4. Infected mosquitoes are collected at required intervals, and stored at –70°C until analysis.

38.3.10 Demonstration of virus transmission

To demonstrate virus transmission, mosquitoes can be allowed to feed on susceptible suckling mice which are monitored for disease development, or saliva may be collected directly from mosquitoes.

(a) Forced salivation

Various methods have been used to collect mosquito saliva (Aitken, 1977; Beaty and Aitken, 1979; Hurlbut, 1966) for analysis (Beaty and Aitken, 1979; Hurlbut, 1966; James, 1994; Racioppi and Spielman, 1987).

1. Deprive mosquitoes of food for 24 h prior to feed.
2. Remove legs and wings.
3. To a 50 μL capillary tube, add 3–5 μL of Cargille type B immersion oil or 10% serum-sucrose solution.
4. Insert the proboscis into the tube. Droplets of saliva will be seen to exude from the proboscis.
5. After 60–90 min, remove mosquitoes and store for virus isolation if required.
6. Remove solution from the capillary tube by centrifugation in a tube containing 100 μL 20% FBS-PBS.
7. Since the procedure cannot be performed under sterile conditions, it is preferable to amplify the secreted virus by inoculating dilutions of the diluent into mosquitoes (or suckling mice) rather than titrating the diluent directly in cell cultures. Following a suitable incubation period, the inoculated mosquitoes are examined by IF (see above).

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School of Medicine, 660 South Euclid Avenue, St. Louis, MO 63110-1093, USA. Dr P.J. Bredenbeek can be contacted at The Institute of Virology, Department of Medical Microbiology, University of Leiden, Postbus 320, 2300 AH Leiden, The Netherlands.

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Part Seven
Cell and Organ Culture

CHAPTER THIRTY NINE

Establishing primary cell cultures from disease vectors and maintenance of continuous cell lines

Colin J. Leake

39.1 INTRODUCTION

Insects and acarines are major vectors of human and animal pathogens ranging in size from helminth parasites down through protozoan parasites to the arboviruses. In order to provide *in vitro* systems to study these organisms, considerable interest has focused on the initiation of primary cell cultures and continuous cell lines from disease vectors. This has led, from initial reports in the late 1960s, to the development of primary cell culture methodology and the subsequent development of a large number of continuous cell lines from both vector and nonvector insects (Hink and Hall, 1989).

A review of the requirements for primary culture initiation from disease vectors reveals that there are many differences in fine detail, but there are also common themes linking the different systems studied. By far the majority of the cell lines from vector species have been established from mosquitoes (Schneider, 1987), and several cell lines have also been established from ticks (Yunker, 1987) which, although strictly not insects, are of major importance to the medical entomologist because of their efficiency as vectors. The aim of this chapter has been to focus on these common themes by summarizing the techniques which our group developed for establishing primary cultures from mosquitoes (Varma *et al.*, 1979, Pudney *et al.*, 1979a), and ticks (Pudney *et al.*, 1979b), extending to a number of established cell lines. Key source references should be consulted for those interested in reviewing work relating to other specific species such as tsetse flies (Schneider, 1979), blackflies (Pudney, 1980), sandflies (Tesh and Modi, 1983), biting midges (Wechsler *et al.*, 1989) and hemipteran bugs (Pudney and Lanar, 1977).

Currently, vector cells have been used extensively in studies on arboviruses *in vitro*. However, work with vector cells for co-cultivation with parasites, physiological experiments, and potential applications in molecular biology have only just begun to be explored. The availability of cell lines from nonvector insects such as *Toxorhynchites* mosquitoes (Kuno, 1981) and the report of *in vitro* development of the

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sporogonic stages of avian malaria in the presence of *Drosophila* fruitfly cells (Warburg and Miller, 1992), suggests interesting avenues may be provided by defined cell populations producing specific molecules essential to parasite development *in vitro*. Over the last 10–15 years, the widespread use of *Drosophila* cells in genetic studies has now advanced from the stage of established cell lines derived from the basic primary culture sources of dissociated embryonic tissue, imaginal discs or larval haemocytes to a wide range of new techniques. Reference should be made to comprehensive discussions of methods, such as mass isolation of *Drosophila* imaginal tissues (Natzle and Vesenka, 1994), fractionation methods for mass isolation and cultivation of specific types of *Drosophila* embryonic cells (Mahowald, 1994), fluorescence-activated cell sorting methods for isolating and cultivating specific *Drosophila* cell types (Cumberledge and Krasnow, 1994), and cloning and expression of exogenous genes using cell transformation techniques applied to established *Drosophila* cell lines (Cherbas *et al.*, 1994). It is an obvious step for these types of approaches to be applied to the study of vector cells and disease organisms.

39.1.1 Selection of tissues

Cells with a high mitotic potential are required to initiate insect primary cultures and embryonic or neonate larval tissues have been the principal source. Fully differentiated tissue has been used much less frequently, with adult mosquito ovary and developing tick nymphal tissue being the most successful choice. Remaining efforts with adult tissue have led to 'organ' culture, in which tissue integrity can be maintained for a relatively short period with little or no cell proliferation (Chapters 40–43).

39.1.2 Cell culture media

The culture media used have been developed along two lines. First, and most frequently, using an empirical approach employing previously described media or modifications of established formulations to new species. Second, the rational approach by designing media specifically for the job on the basis of analysis, i.e. haemolymph analysis. In initial reports, insect haemolymph was frequently used as a media supplement, but a key advance was made in the late 1960's when it was established that fetal calf serum, commonly used in vertebrate cell culture, was an excellent replacement for haemolymph additives. In general, insect cell culture media can be described as relatively simple salt solutions with a high sodium/potassium ratio, a multiple protein/amino source provided by lactalbumin and yeastolate, and fetal calf serum providing a variety of other materials. For many species we now know that primary cultures and cell lines can be established and grown in defined vertebrate cell culture media or specific, serum-free, formulations, although, from a practical point of view, it is relatively easy to adapt established lines to a wide range of media.

39.1.3 Culture conditions

(a) Sterility

The initial problem to be surmounted is to ensure microbiological sterility. This has been achieved by washing tissue sources, usually eggs, with various quite toxic

chemicals, preferably with wetting agents, for short periods of time to remove and kill the surface contaminating bacteria, yeast and fungi. Subsequent steps in handling requires the use of full sterile techniques usually backed up during the early stages of primary culture by the routine addition of high levels of antibiotics such as penicillin and streptomycin to the culture media. Fungal contamination can be a considerable problem as incubation temperatures and tissue culture media are ideal for fungal growth. Fungi are quite difficult to deal with chemically as the inhibitory doses of commonly used anti-fungal agents such as fungizone® and mycostatin® are usually quite toxic to sensitive primary cultures. Surface sterilization methods are also ineffective against viruses and it is quite possible to have persistent unapparent insect virus and/or arbovirus contaminants in these cells which may be subcultured long-term and interfere with detailed studies.

(b) Cell culture environment

Most primary cultures have tended to be initiated in media in sealable glass containers usually in air, rather than with any special gaseous requirements. Although there is a huge selection of tissue culture plasticware available, these materials have largely been developed for use in the mammalian cell culture market and surfaces are treated and optimized using mammalian cells. Experience has shown that plastics can prove to be quite variable in suitability for growing insect cells and it is recommended that plastics are best reserved for established cell lines.

Typically for vector cells an incubation temperature of 28°C is the norm, although these cells will survive quite happily at RT, or lower for extended periods. Higher temperatures can be tolerated for short periods of time, although heat shock proteins will be produced. Vertebrate cell culture temperatures of 37°C will be lethal if extended.

39.2 MATERIALS

39.2.1 Mosquito and tick primary culture

1. Laboratory colonies of mosquitoes and ticks are required to provide freshly laid mosquito and tick eggs, or developing tick stages.
2. Sterile PBSA: dissolve pre-weighed tablets of Dulbecco's phosphate buffered saline A in the recommended volume of ddH_2O and sterilize by autoclaving at 10 lb/in^2 for 10 min.
3. Sterile Mitsuhashi and Maramorosch (MM): medium available commercially or fabricated from raw materials (Section 39.2.2).
4. Sterile Modified Kitamura's medium: fabricated from raw materials (Section 39.2.2).
5. Sterile Varma and Pudney's VP12 medium: fabricated from raw materials (Section 39.2.2).
6. Sterile Leibovitz L-15 medium: available commercially or reconstituted from commercially available powder (Sections 39.2.2 and 39.4.1).
7. Sterile H-LAC medium: available commercially (Section 39.2.2).
8. Sterile fetal bovine serum: available commercially. Heat inactivated at 56°C for 30 min.
9. Penicillin (10 000 U/mL) and streptomycin (10 000 μg/mL) concentrate.

10. White's solution: to make 1 L dissolve 0.25 g HgCl and 6.0 g sodium NaCl in 750 mL glass ddH_2O. Carefully add 125 mL conc HCl and then 125 mL ethyl alcohol. Store at RT in 500 mL glass screw capped bottles.
11. Rinaldini's salt solution (RSS): dissolve 8 g NaCl, 0.2 g KCl, 0.05 g $NaH_2PO_4.H_2O$, 1 g D-glucose, 1 g $NaHCO_3$, 0.676 g $Na_3C_6H_5O_7.2H_2O$ in 1 L of ddH_2O. Sterilize by autoclaving at 10 lb/in^2 for 10 min. Decant into glass bottles and store at 4°C.
12. Roccal®: Surface disinfectant containing benzalkonium chloride (Winthrop Laboratories Ltd., Surbiton, UK).
13. Sterile O.25% trypsin solution.
14. Sterile glass Leighton tubes.
15. Sterile policemen: autoclaved glass rods that have their end bent in a gas flame and covered with silicon rubber tubing. Used manually to detach cells from the tissue culture flasks. Fine glass rods can be easily fabricated for use in Leighton tubes.
16. Selection of sterile stainless steel dissection instruments. Specifically, fine spring scissors, curved forceps, mounted dissecting needles and a bacteriological wire loop.
17. Small metal dissecting dish containing wax for tick dissections.
18. Sterile entomological pins.
19. Sterile glass rods with ends flattened into coin-shaped disc for crushing tick eggs.
20. Selection of sterile tissue culture glassware or plastics; Petri dishes, centrifuge tubes.

39.2.2 Media preparation (Note 2)

The principal media used are shown in Table 39.1 (with the exception of Leibovitz L-15, Section 39.4.1). Several formulations, such as MM and H-LAC, are available commercially as sterile liquid, or powdered preparations (L15), or can be fabricated from raw materials and sterilized. All chemicals used to prepare media are Analar grade or higher and at least single-glass ddH_2O is used.

1. Weigh individual chemicals and dissolve in the order shown in Table 39.1 or according to manufacturer's instructions (L-15) with continuous stirring on a magnetic stirrer.
2. Allow to standard for up to 2 h at RT.
3. Adjust pH of the media to 6.9 with slow drop-by-drop addition of 2% KOH solution accompanied by simultaneous thorough mixing on a magnetic stirrer. This is particularly important with VP12 medium as irreversible precipitation occurs if KOH is added to quickly.
4. Sterilize by positive-pressure filtration, using a compressed nitrogen gas source, through a pre-sterilized 0.22 μm membrane filter apparatus and dispense into sterile glass bottles prior to storage at 4°C.
5. Incubate 10 mL aliquots of medium with 10 mL sterile tryptose phosphate broth at RT and 37°C for several days to confirm sterility.
6. For use in early stages of primary culture preparation add 10× standard tissue culture concentrations of antibiotics to a final concentration of 1000 U/mL of penicillin and 1 mg/mL of streptomycin respectively.

Table 39.1 Formulation of principal cell culture media

	MM (mg/L)	*MK* (mg/L)	*VP12* (mg/L)	*HSU* (mg/L)	*MHSU* (mg/L)	*H-LAC* (mg/L)
NaCl	7000	8125	3900	4500	1575	8000
$NaH_2PO_4.H_2O$* or $2H_2O$†	200*	–	550†	–	–	–
$MgCl_2.6H_2O$	100	–	1100	–	–	100
$MgSO_4.7H_2O$	–	–	1200	400	140	100
KCl	200	625	550	800	280	400
$CaCl_2$ (anhydrous)	–	–	–	350	122.5	140
$CaCl_22H_2O$	200	125	400	–	–	–
KH_2PO_4 (anhydrous)	–	125	–	250	87.5	60
D-Glucose	4000	–	2000	1600	560	1000
Sucrose	–	–	–	6000	2100	–
Choline chloride	–	–	250	–	–	–
Inositol	–	–	400	–	–	–
$NaHCO_3$	120	125	500	1000	350	350
$Na_2HPO_47H_2O$	–	–	–	–	–	90
Lactalbumin hydrolysate	6500	8125	5000	20000	8650	6500
Yeastolate	5000	625	–	2000	3200	–
Bacto-peptone	–	–	–	5000	1750	–
Bovine albumin fraction V	–	–	1000	–	–	–
5% Glutamine solution	–	–	6ml	–	–	–
L-Malic acid	–	–	–	600	210	–
α-Ketoglutaric acid	–	–	–	400	140	–
Succinic acid	–	–	–	60	21	–
Fumaric acid	–	–	–	60	21	–
Eagle's Vitamin Mixture 100×	–	–	20ml	–	–	–
Phenol red	–	–	–	–	–	10ml
Medium 199 (10×)	–	–	–	2ml	50ml	–
Whole egg ultrafiltrate	–	–	–	–	10ml	–
Fetal bovine serum	20%	20%	10%	10%	10%	10%

MM, Mitsuhashi and Maramorosch's medium; MK, modified Kitamura's leafhopper medium; VP12, Varma and Pudney's tick cell culture medium; HSU, 721 medium for *Culex quinquefasciatus* ovarian cell culture; MHSU, modified 721 medium for *Culex tritaeniorhynchus* ovarian cell culture; H-LAC, lactalbumin hydrolysate with Hanks' salts.

7. For care and maintenance of primary cultures and established cell lines antibiotic concentration should be reduced immediately after initiation. This is achieved during care of the primary by replenishing old media with fresh media containing a standard tissue culture dose of 100 U/mL penicillin and 100 μg/mL of streptomycin respectively (Section 39.3.2c).
8. Prior to use, add fetal bovine serum as indicated in Table 39.1.
9. Complete L-15 medium is routinely supplemented with penicillin and streptomycin as above, 10% tryptose phosphate broth, and 10% fetal bovine serum.

39.3 METHODS (NOTE 2)

39.3.1 Mosquito primary cell culture (Notes 3 and 4)

(a) Tissue sources

Source materials are usually obtained from colony mosquitoes.

1. Egg batches laid 24–36 h previously from Culicine species that lay drought-resistant eggs on filter paper, i.e. *Aedes* and *Haemagogous* species mosquitoes.
2. Floating eggs or egg rafts laid 24–36 h previously that are not desiccation resistant, i.e. *Anopheles* species and *Culex* species mosquitoes.
3. Ovarian tissue dissected from 3-day-old adult female *Culex* mosquitoes.

(b) Surface sterilization of Aedes aegypti *eggs (Pudney et al., 1979a)*

1. Remove all debris from dried eggs on filter paper. Cut into small pieces.
2. Wash twice in acetone and immerse in White's solution for up to 20 min.
3. Put eggs in RSS and place container in vacuum desiccator and reduce pressure to induce hatching. Eggs should hatch synchronously within 1 h and should be used immediately.

(c) Alternative method for Aedes aegypti *eggs (Pudney et al., 1979a)*

1. Remove debris from dried eggs on filter paper. Cut into small pieces.
2. Add 10% solution of Roccal® in ddH_2O. Wet eggs by pipetting and stand for 15 min.
3. Remove Roccal® and wash twice in sterile ddH_2O and once in PBSA containing 10× antibiotics (Penicillin 1000 U/mL, streptomycin 1 mg/mL).
4. Place eggs in clean PBSA with 10× antibiotics and induce to hatch as above.

(d) Surface sterilization of eggs from anopheline mosquitoes (Pudney et al., 1979a)

1. Remove floating eggs from egg bowls with bacteriological wire loop and transfer to 1:5 solution of Roccal® solution in a Petri dish.
2. Immerse eggs with vigorous pipetting for 4 min.
3. Remove Roccal® with any sunken eggs (which will not hatch) and wash eggs twice in ddH_2O with 10× antibiotics as above.
4. Remove eggs with a sterile wire loop to a Petri dish containing sterile ddH_2O. Cover with foil and leave at 28°C overnight. Several hundred larvae will emerge overnight.

(e) Surface sterilization of adult Culex *mosquitoes and removal of ovarian tissue (e.g.* Culex quinquefasciatus, *Hsu* et al., *1970,* Culex tritaeniorhynchus, *Hsu* et al., *1972)*

1. 2–3 day-old adult female mosquitoes are immobilized by stunning and then immersed in 0.25% mercuric chloride in 70% ethyl alcohol for 2 min.
2. Wash twice in sterile ddH_2O and place mosquito on a sterile glass slide in a few drops of HSU's medium for *Culex quinquefasciatus* and modified Hsu's medium for *Culex tritaeniorhynchus*.

3. Make an incision in the middle of the third abdominal segment and remove ovaries to a culture tube with HSU's medium for *Culex quinquefasciatus* and modified HSU's medium for *Culex tritaeniorhynchus*. No further tissue dissociation has been reported. Sealed Leighton tubes are placed at a 20° slant in a 28°C incubator.

(f) Dissociation of mosquito larval tissues

1. Freshly hatched larvae are immobilized by transferring them to a sterile centrifuge tube which is placed vertically in an ice bath. The chilled larvae sink to the bottom of the centrifuge tube and the excess water or saline removed.
2. Remove pelleted larvae to a small Petri dish in a few drops of 0.25% trypsin solution.
3. Cut up all the larvae with sterile fine spring scissors until no moving larvae can be seen.
4. Remove the larval pieces into 4 mL 0.25% trypsin solution in a centrifuge tube and incubate at 35°C for 10 min.
5. Pipette vigorously with a Pasteur pipette and centrifuge at 125 g for 10 min.
6. Remove supernatant fluid and resuspend the pellet in 5 mL of RSS or PBSA with 10× antibiotics, recentrifuge. Repeat.
7. Remove supernatant fluid and resuspend pellet in following complete media with 10× antibiotics (Table 39.1): *Aedes aegypti* and *Aedes pseudoscutellaris* (i) MM, (ii) 1:1 MM/VP12, (iii) L-15. *Anopheles stephensi* and *Anopheles gambiae* 1:1 MK/VP12.

(g) Seeding and care of mosquito primary cultures

1. The day before seeding of primary cultures, condition the surfaces of clean Leighton tubes by placing 1 mL of the required fresh complete medium in the tube. Discard prior to seeding.
2. The methods described produce a mixture of cells and larval fragments for seeding. A high seeding density is the target (100–150 processed larvae/mL of medium) as low density cultures will be nonproductive or very slow. Seed 1 mL suspensions into replicate glass Leighton tubes and incubate at 28°C.
3. Do not disturb the primary culture for several days. Then observe regularly using an inverted tissue culture microscope.
4. Media changes: During the early stages, approximately every 7 days, stand the tube vertically for a few min to allow tissue clumps to settle and then carefully aspirate approximately the top half of the supernatant medium leaving the tissue behind. Replace this half of the old medium with fresh medium containing a standard tissue culture dose of 100 U/mL penicillin and 100 μg/mL of streptomycin respectively.
5. Promising cultures will contain numerous actively twitching larval pieces with clumps of tissue and single cells attached to the glass. If after 3 months there is no sign of this the culture can be discarded as a failure (Note 3).
6. In viable cultures, several morphological cell types will become apparent progressively growing out from the attached clumps, and hollow balls of cells will grow out from the floating larval pieces. These may attach and collapse to form attached patches or produce bunches of floating vesicles.
7. Re-seeding: When about half the container is covered with cell patches it will be necessary to re-seed the tube to ensure a smoother distribution of cells and avoid dengeneration in the cell clumps. Remove all the medium and floating tissue clumps to a centrifuge tube. Allow the tissue clumps to settle or gently cen-

trifuge. Remove half the supernatant medium and discard it. To the original tube add half the original volume of fresh medium routinely containing standard tissue culture doses of penicillin (100 U/mL) and streptomycin (100 μg/mL) and detach the cells from the glass by using a fine glass policeman and with gentle pipetting. Add the tissue clumps and old medium from the centrifuge back to the original tube. Return to the incubator.

8. Subculture: Repeat weekly medium changes and re-seeds as necessary until a complete monolayer has formed. At this stage add an equal volume of fresh medium and detach the cells as above and dispense into two tubes. This constitutes a 1:2 split and the culture then technically becomes a cell line at subculture number 1.

39.3.2 Tick primary culture (Note 5)

(a) Tissue source: developing adult tissues of Rhipicephalus appendiculatus *ticks*

Engorged nymphs are used when the developing adults are seen within the nymphal coat. This stage is reached between 8 and 19 days after dropping off the host animal, dependent on temperature.

(i) Surface sterilization of nymphs and preparation of tissues

1. Remove debris by washing nymphs once in ddH_2O and twice in 10% Roccal® in ddH_2O and then stand in 10% Roccal® for 20 min.
2. Rinse briefly in ethyl alcohol and twice in sterile ddH_2O with 10× antibiotics before placing in a small Petri dish containing PBSA with 10× antibiotics.
3. Sterilize paraffin wax in a small metal dissecting dish by partially heating the wax from the top with a flame to melt and then allowing it to set by cooling. A shallow depression will result in the wax which is filled with PBSA with 10× antibiotics.
4. Transfer surface sterilized adult to the wax dish and pin to the wax anteriorly and posteriorly, avoiding the gut, using sterile entomological pins.
5. The dorsal integument is then removed by cutting around the body with fine spring scissors and the entire alimentary canal removed using forceps and fine dissecting needles.
6. All the remaining tissues: epidermis, legs and central ganglion, are then removed, washed twice in PBSA with antibiotics and then transferred to a clean Petri dish containing H-LAC medium (Table 39.1).
7. Multiple dissections are completed and pooled.

(ii) Dissociation of nymphal tissues

1. The pooled tissue is then washed twice in PBSA and the tissues cut up as finely as possible with spring scissors in a few drops of 0.25% trypsin solution in a centrifuge tube and incubated at RT for 10 min.
2. Pipette vigorously with a Pasteur pipette and centrifuge at 125 g for 10 min.
3. Remove supernatant fluid and resuspend the pellet in 5 mL PBSA with antibiotics, recentrifuge. Repeat.

7. Remove supernatant fluid and resuspend pellet in complete in H-LAC or L-15 medium.
8. Seed primary culture (Section 39.3.2c).

(b) Tissue source: embryonic tissues of Boophilus *microplus ticks*

Take fully fed adult female hard ticks 1–2 days after dropping off host animal.

(i) Surface sterilization of ticks and preparation of embryonic tissues

1. Surface sterilize whole female tick by repeating 3.2.1.1 steps 1 and 2.
2. Dry female on sterile filter paper and place into a sterile Petri dish in a humidified box at 28°C and allow to oviposit, usually commencing 1–2 days later.
3. Record time from oviposition and use eggs 12–13 days old.
4. Remove eggs using sterile curved forceps to a sterile glass Petri dish.
5. Add sufficient H-LAC medium with 10× antibiotics to hold the eggs together by surface tension, but not to allow them to float. Using a sterile glass rod with the end flattened into a circular disc, or the flattened plunger of a sterile glass syringe, gently crack open the egg shells.
6. Remove the eggshells by filtering the mixture of tissue and eggshells through a sterile 100–120 μm sintered glass funnel.
7. Pellet the tissue by centrifugation at 250 **g** for 10 min.
8. Discard supernate and wash twice in fresh H-LAC with 10× antibiotics before resuspending pellet in fresh H-LAC medium with 10× antibiotics and seeding the primary cultures (Section 39.3.2c)

(c) Seeding and care of tick primary cultures (Note 5)

1. Procedures are identical to mosquito methods (Section 39.3.1g) with substitution of H-LAC or L-15 medium with 10× antibiotics as required.
2. Use tissues at a rate of 10 nymphs/mL of medium and eggs of 2.5 females/mL of medium. Seed 1 mL aliquots into glass Leighton tubes.
3. During routine media changes, re-seeds and subcultures, the media used should contain standard doses of penicillin and streptomycin.

39.3.3 Maintenance of established mosquito and tick cell lines

1. There is no substitute for experience in handling cell cultures. It is of paramount importance to observe cultures on a daily basis and to keep a detailed written log of all observations. This will provide both short and long-term information on the health of the cells and help to identify problem areas (Note 5).
2. Generally, the expectation will be that cells will remain quite firmly attached to the glass until a quite heavily confluent cell sheet has been formed. Media pH, initially around 7.0–7.2, will become more acidic. Phenol red indicators in HLAC and L-15 will progressively turn orange, and then yellow, if cells are actively dividing and reducing the pH of the medium.
3. Failure of the cells to attach, or early detachment of the cells, or slow growth of the cells, will result initially in little change in the colour of the phenol red indi-

cator in the media, followed later by the development of a pink colour in the indicator which indicates major problems as the pH is becoming too alkaline. Complete changes of media may help to cure the problem.

4. Once the cells have reached a fully confluent monolayer, subculturing becomes necessary, and eventually will evolve to a routine weekly maintenance of stock cultures.
5. An initial subculture has been described above (Section 39.3.1g, step 8; Note 6). All cells are routinely detached from the glass using rubber policemen (Note 7).
6. Mosquito cell lines will usually accelerate their growth quite quickly. This will become apparent, in that monolayers will form more quickly and it will be necessary to subculture the cells at periods of less than 1 week's incubation. At this point it is likely that the cells will have been subcultured about 4 or 5 times and the number of Leighton tubes will have expanded to about 10; it is desirable to move up in scale to small bottles.
7. Take five confluent tubes, remove half the media and add fresh media to the original volume. Detach the cells, gently pipette to break up cell clumps and dispense to pre-conditioned 1 oz medical flat bottles closed with silicon bungs. Incubate. When confluent split as necessary.
8. Adjust the split ratio of the cells by adding sufficient fresh medium to form confluent monolayers in the required number of containers after 1 week's incubation. Mosquito cells usually grow very rapidly when fully established, whereas tick cells are very slow (Note 8).
9. The ratio of old to new medium provided should be decided by the health of the cells. For mosquito cells it is practical to reduce the proportion of old to new medium rapidly to complete replacement with fresh media. Slow growing tick cells often require a proportion of old medium virtually continuously and will show marked deterioration if replenished with excessive fresh medium.

39.3.4 Storage of cells in liquid nitrogen

1. As soon as possible, it is essential to establish a depository of cells in liquid nitrogen storage (Note 9).
2. Select cultures that are growing well and are just approaching a confluent monolayer.
3. Remove all the old media and resuspend and pool contents of 5 × 1 oz medical flat bottles in 5 mL of fresh culture medium supplemented with 10% membrane filter sterilized dimethyl sulfoxide (DMSO). Dispense complete contents to 5 × 2 mL sterile screw cap cryotubes.
4. Suspend the tubes in liquid nitrogen vapour for 3 h using a proprietary liquid nitrogen cell freezing attachment adjusted to give a cooling rate of 1°C/min.
5. Rapidly immerse tubes in marked canes in liquid nitrogen.
6. Shortly after storage, remove a single cryotube from the stock, thaw rapidly and resuspend entire contents of tube with 4 mL of fresh medium into a pre-conditioned culture bottle. A high proportion of the cells should attach to the glass rapidly. After 24 h, completely change the medium to remove toxic DMSO. Incubate. Subculture as necessary.
7. In the event of poor, or no attachment, test other ampoules, or discard whole batch of stored cells. Repeat storage procedure using a fresh batch of cells.

NOTES

1. Media: Liebovitz L-15 medium is best purchased commercially as it has a complex formula and would be very expensive to fabricate from raw materials in small quantities.
2. Facilities: It is assumed that full facilities for tissue culture are available, in addition to standard laboratory equipment, and that throughout all procedures will employ full sterile technique. All methods are performed in a positive pressure or downflow recirculation type tissue culture workstation which provides a sterile laminar air-flow environment but should not be used for pathogen work. Full supporting glassware washing and sterilization facilities, cooled incubators and refrigerator/freezer storage space and liquid nitrogen storage are required. Inverted tissue culture microscope and zoom dissecting microscope are required. This represents a substantial initial capital cost.
3. Time: Initiation of new insect primary cultures is a laborious and time-consuming task. Primary insect cell cultures are quite unlike comparable vertebrate primary cultures in terms of the speed of establishment of monolayers of cells and subsequent progression past the first subculture. This period can be many weeks or even months and it is therefore recommended that the use of an existing established system should be explored initially.
4. Existing cell lines: If there is an interest in working with a specific cell system the rational approach of preparing cell lines from a major vector may well be of limited practical use. Many cell lines being produced from *Aedes aegypti*, the principal epidemic vector of yellow fever and dengue viruses, have not proved very useful in the study of arboviruses *in vitro*. Only three mosquito cell lines of the 70+ lines produced have found extensive use in virus studies. These are: Igarashi's clone C6/36 of Singh's *Aedes albopictus* cell line (Chapter 36); the *Aedes pseudoscutellaris* cell line established by Varma *et al.* (1974); and cell lines established from the nonvector mosquito *Toxorhynchites amboenensis*, particularly the TRA-284-SF serum-free line (Kuno, 1981).
5. Tick cells: Primary tick cell cultures and tick cell lines are particularly slow and difficult to maintain and require great care, attention to detail, and experience. It may take many weeks or months before the first subculture can be made.
6. Routine subculturing: It is a sensible policy to avoid subculturing or changing the media in at least two confluent bottles of cells weekly. The remaining cultures can then be processed with the confidence that should major contamination or toxicity affect the cells a fall back reserve is always available.
7. Enzymes: Detachment of cells using proteolytic enzyme treatment, such as trypsin, has not routinely been used for mosquito or tick cells and should be avoided.
8. Split ratios: As examples, the weekly split ratios of the *Aedes pseudoscutellaris* (LSTM-AP-61) cell line in MM/VP12 medium at subculture (sc) 10 was 1:3, sc 50 1:12, sc 100 1:22, sc 150 1: 40, sc 200 1:40, sc 300 1:24 (note drop at this time), sc 400 1:50, and of an *Anopheles gambiae* cell line (LSTM-AG-55) in MK/VP12 medium at sc 10 was 1:9, sc 50 1:56, sc 100 1:150, sc 200 1:230, sc 300 1:360, sc 400 1:480, sc 483 1:800. High split ratios are achieved by dilution of a cell suspension, i.e. a 1:800 split of *Anopheles gambiae* cells was achieved by suspending the monolayer in 10 mL of medium and then adding 1 mL of this cell suspension to 7 mL of

fresh medium in a separate bottle. 0.1 mL of this suspension is then seeded to the final container in a final volume of 3–5 mL in a 1 oz medical flat bottle. In contrast, the growth of the *Rhipicephalus appendiculatus* (LSTM-RA-243) tick cell line has typically required a routine split ratio of 1:2 over nearly 9 years of continuous culture (398 subcultures). A split ratio of 1:3 was the maximum ever recorded over a short period when the cells were growing particularly well.

9. Storage of cells: Mosquito cells apparently have an indefinite storage time in liquid nitrogen; currently at least 25 years in our hands. Tick cell storage has not proved successful in the long-term. Cells resuscitated from liquid nitrogen may initially be rather slow and will require care. Do not increase the split ratio to the original rate too quickly.

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CHAPTER FORTY

Culture and analysis of insect salivary glands

Alison C. Morris and Anthony A. James

40.1 INTRODUCTION

Insect salivary glands have proved to be important sources of information in the study of development and pathogen transmission. Studies involving the polytene chromosomes of dipteran larval glands have produced a wealth of information regarding gene expression and genomic mapping. For the most part, polytene chromosomes are prepared for analysis directly from extirpated salivary glands. However, for questions concerned with induction of gene expression in *Drosophila*, an *in vitro* culture system was devised (Ashburner, 1972). Culturing the glands enables the application of strictly controlled conditions for induction. Molecular genetic studies of metamorphosis and development in *Drosophila* have been greatly facilitated by studies of puffing patterns in polytene chromosomes in response to ecdysterone treatment of cultured larval salivary glands (Cherbas, 1993). In vector Diptera, adult salivary glands can serve as a major route of transmission of arthropod-borne pathogens. As such, they are compelling objects of study in the investigation of host/parasite interactions. The mechanisms involved in parasite invasion of the insect salivary glands, for the most part, are unknown, but constitute the focus of much research as transmission blocking strategies are evaluated for use in disease control (Touray *et al.*, 1992). In addition, the evasion of host immune responses and haemostasis in blood-feeding (Ribeiro, 1987) raises a fascinating issue of the role of the salivary gland in parasite transmission.

Molecular analysis of salivary gland-specific gene expression may elucidate the precise mechanisms involved in parasite invasion and transmission to the vertebrate host. For example, exogenous genes may be expressed in the salivary glands to determine their role in blocking parasite invasion or replication in the glands. Such exogenous gene expression may result in unusual presentation of surface receptors required for invasion, or expression of antisense viral RNA to block replication of pathogenic viruses (Carlson *et al.*, 1995). However, despite the progress made in the development of putative transmission blocking constructs, stable germline transformation, which would facilitate exogenous gene expression, has not yet been demonstrated in nondrosophilid insects (Handler and O'Brochta, 1991). In the absence of such a system, exogenous gene expression studies may be carried out using transient assays on salivary glands in culture.

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Although polytene chromosomes have been used in species identification of *Simulium* (Brockhouse *et al.*, 1993), and in genomic mapping of *Anopheles gambiae* (Zheng *et al.*, 1991), endogenous gene expression studies in cultured glands have not been reported in insects other than *Drosophila*.

In devising strategies to assess gene expression in the salivary glands, developmental and sexual dimorphisms of holometabolous, haematophagous insects must be considered. In general, the salivary glands of holometabolous insects differ structurally between the adult and larval stages. Similarly, female salivary glands of haematophagous insects are larger and more complex than those of the male. For example, larval salivary glands of the mosquito, *Aedes aegypti*, consist of a single cylindrical lobe, dilated at the distal end (Jensen and Jones, 1957). In contrast, the adult glands are made up of three lobes, two lateral, and a shorter, wider, median lobe. The lateral lobes are divided into two distinct regions, proximal and distal, the former producing enzymes involved in sugar feeding and the latter implicated in blood-feeding (James, 1994). The male salivary glands are distinctly smaller than those of the female and overlap the morphology and function of the female proximal lateral lobes. Each lobe is made up of cells which enclose an apical cavity and are distributed radially around a salivary duct. The lobes are bounded by a basal lamina consisting of protein, glycoprotein and mucopolysaccharides (Levinson and Bradley, 1984).

In *Ae. aegypti*, salivary glands have been successfully transfected with exogenous DNA (Morris *et al.*, 1995) using a liposome-mediated transfection reagent, DOTAP. DOTAP is a cationic lipid that forms liposomes in aqueous solution. These liposomes interact with DNA to form stable complexes that adhere to the cell surface, fuse with

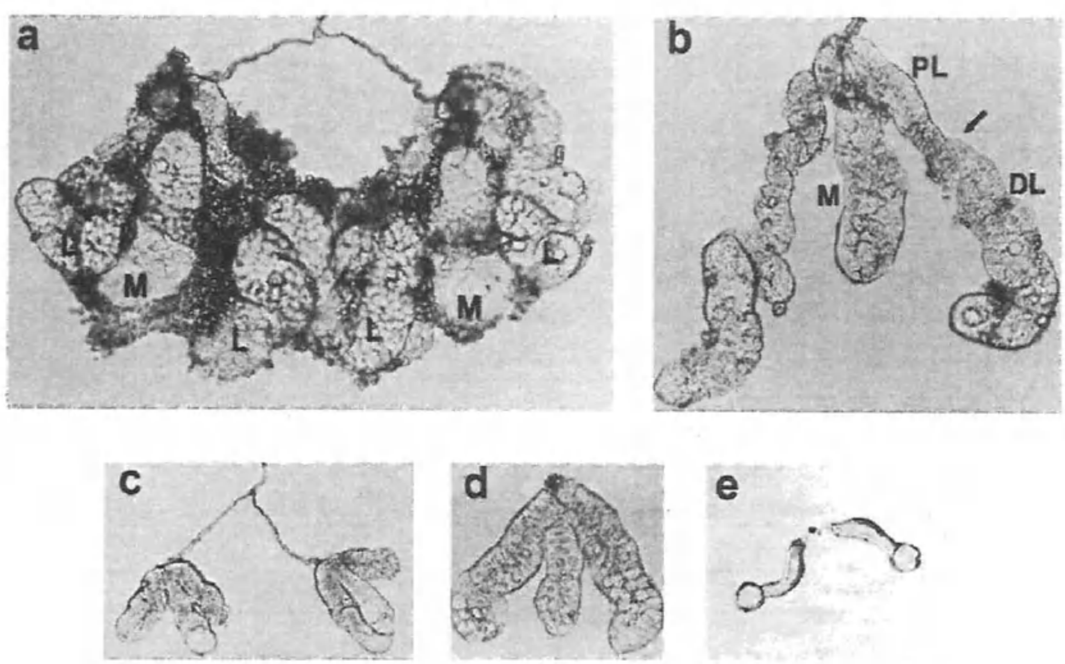


Figure 40.1 Salivary glands of *Ae. aegypti*. (A) A pair of adult female salivary glands connected to the common salivary duct. The dark globular material is fat body. (B) A single adult female salivary gland. The arrow indicates the intermediate region of the glands between the proximal lateral lobe (PL) and the distal lateral lobe (DL). (M, medial lobe.) (C) A pair of adult male salivary glands. (D) A single adult male salivary gland. (E) A pair of larval salivary glands. The glands in (A), (C) and (E) were photographed and reproduced at the same magnification.

the cell membrane and release the DNA into the cytoplasm. The basement membrane appears to act as a barrier to liposome fusion in transfection experiments but is readily removed by treatment with 0.2% elastase. In addition, metabolic labelling may be used to monitor protein synthesis and turnover in salivary glands.

To date, salivary gland transfection experiments have been used to assay putative salivary gland-specific promoter sequences (Morris *et al.*, 1995). Due to the small number of cells available for transfection, an extremely sensitive reporter gene is required. In experiments with cultured mosquito glands, the firefly luciferase gene was determined to be the optimum reporter based on the high sensitivity of its detection and low signal to noise ratio.

The methods described below have been successfully used to culture and transfect adult salivary glands of *Ae. aegypti*.

40.2 MATERIALS

1. *Aedes* saline: (Koller *et al.*, 1989) 150 mM NaCl, 4 mM KCl, 3 mM $CaCl_2$, 1.8 mM $NaHCO_3$, 0.6 mM $MgCl_2$ buffered with 25 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethansulfonic acid) pH 7 at 27°C.
2. Reddy's medium: (Table 40.1; modified from Bohm *et al.*, 1978 and H.H. Hagedorn, unpublished results).

Table 40.1 100 ml modified Reddy's Medium

Solution A (75 ml)	*mM in final medium*	*MW*	*mg*
L-Alanine	5	89.1	44.6
L-Arginine · HCL	5	210.7	105.4
L-Asparagine (desiccate)	5	132	66
L-Aspartic acid	5	133	66.5
L-Cysteine · HCl · H_2O	2	157.6	31.5
L-Glutamic acid (desiccate)	5	147.1	73.5
L-Glutamine	5	146	73
L-Glycine	10	75	75
L-Histidine · HCl · H_2O	15	209.7	314
L-Isoleucine	2	131	26.2
L-Methionine	2	149	29.8
L-Phenylalanine	2	165	33
L-Proline	5	115	57.5
L-Serine	10	105	105
L-Threonine	2	119	23.8
L-Tryptophan	2	204	40.8
L-Tyrosine	1	181	18.1
L-Valine	2	117	23.4
PTU (phenylthiourea)	16	152.2	2.5
PVP (polyvinylpyrolidine) -40	500	40×10^3	2000

Stir for 1 h at room temperature

Solution B (5 ml)	*mM in final medium*	*mg*
L-Lysine · HCl	5	91.5
L-Leucine	1	13.1

Table 40.1 (*continued*)

Solution B′ (10 ml, use 1 ml in final 100 ml medium	*mM in final medium*	*mg*	
Uracil	0.025	2.8	
Adenine	0.050	6.8	
Guanine	0.050	7.6	
Cytosine	0.050	5.6	
Thymine	0.025	3.2	
Note: if this solution does not dissolve, add NaOH (6 M) until it does, *then* make up to volume.			
Solution C_I (5 ml)	*mM in final medium*	*mg*	
NaCl	25	140	
KCl	1	7.4	
$MgCl_2 \cdot 6H_2O$	0.6	12	
$NaHCO_3$	3.0	25	
Solution D (prepare fresh, 5 ml)	*mM in final medium*	*mg*	
K_2HPO_4	2	34	
HEPES	25	595	
'D+'-Glucose	25	450	
Solution C′ (2 ml)	*mM in final medium*	*MW*	*mg*
$CaCl_2 \cdot 2H_2O$	1.7	147	24.9
Pen (1 ml)	*mM in final medium*	*MW*	*mg*
Penicillin-G (1650 units/mg)	0.4	356.4	15
Strep (1 ml)	*mM in final medium*	*MW*	*mg*
Streptomycin sulfate	0.17	1457.4	25

Mix solutions A, B, B′, C_I and D. Adjust pH to 6.8–7.0 with 6 M NaOH (use dilute HCl if necessary). Stir in solution C′ slowly. Add pen and strep solutions. Adjust volume to 100 ml with ddH_2O and filter through a 0.2 µm filter.

3. Reddy's medium minus cysteine and methionine.
4. 0.2% elastase: (elastase available from Sigma Chemical Company, St. Louis, MO, USA) prepared in *Aedes* saline.
5. DOTAP: N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethyl-ammoniummethylsulfate (Boehringer Mannheim, Indianapolis, IN, USA).
6. Plasmid DNA: for transfection should be prepared as for DNA used in microinjection experiments (Chapter 35) and resuspended in TE (10 mM Tris pH 7.5, 1 mM EDTA, pH 7.5)

7. 1.5 mL disposable Kontes tubes and pestles: (Kontes Scientific Glassware/ Instruments, Vineland, NJ, USA).
8. 10% sucrose solution: containing penicillin G (200 U/mL), streptomycin disulfate (200 μg/mL) and amphotericin B (0.5 μg/mL).
9. ^{35}S translabel: containing ^{35}S-labelled cysteine and methionine (ICN Biochemicals, Irvine, CA, USA).
10. ENLIGHTNING™ (DuPont NEN® Research Products, Boston, MA).

40.3 METHODS

40.3.1 Culture of mosquito salivary glands

1. Bacterial contamination associated with salivary glands may be reduced by feeding mosquitoes on a 10% sucrose solution with penicillin G (200 U/mL), streptomycin disulfate (200 μg/mL) and amphotericin B (0.5 μg/mL) for 3–5 days prior to dissection.
2. Dissect salivary glands in a drop of *Aedes* saline from 3–5 day-old adult mosquitoes and maintain on ice in 100 μL *Aedes* saline.
3. Glands may be transferred to Reddy's medium either by picking up individual glands with a dissecting needle, or by removing the *Aedes* saline from the glands using a finely drawn Pasteur pipette and replacing with Reddy's medium. In large scale experiments involving multiple samples and glands, glands may be concentrated by spinning for 5 s in an Eppendorf benchtop microcentrifuge. The *Aedes* saline can then be removed and replaced by Reddy's medium.

40.3.2 Transfection of salivary glands

1. Dissect 50 pairs of salivary glands from 3–5 day-old adult mosquitoes. Maintain on ice in 100 μL *Aedes* saline in a 1.5 mL polypropylene microcentrifuge tube.
2. Concentrate the glands by centrifugation for 5 s at full speed in an Eppendorf benchtop microcentrifuge.
3. Remove the *Aedes* saline and replace with 200 μL of 0.2% elastase in *Aedes* saline (Note 1). Incubate at RT for 45 min.
4. Wash the glands 3× with 1 mL of *Aedes* saline by concentrating the glands as in step 2. Resuspend the glands between each step by gently tapping the tube.
5. Resuspend the glands in 500 μL of Reddy's medium.
6. Prepare a DOTAP/DNA cocktail in a 5 mL polystyrene culture tube: Dilute 16 μg of DOTAP (1 mg/mL) up to 50 μL with ddH_2O. Dilute 20 μg of DNA up to 50 μL with ddH_2O. Mix the DOTAP and DNA solutions and incubate at RT for 10 min. Add 150 μL of Reddy's medium. It is recommended to optimize the concentration of transfection reagent, the DNA and the time of incubation for every system.
7. Using a sterile Pasteur pipette, transfer the glands in Reddy's medium into the DOTAP/DNA cocktail. Rinse the microcentrifuge tube with an additional 250 μL of Reddy's medium and add this to the cocktail to give a final volume of 1 mL.
8. Incubate the glands at 27°C for 18–20 h.
9. Remove the DOTAP from the glands by transferring the reaction mix to a polypropylene microcentrifuge tube using a glass Pasteur pipette, and concentrate the glands by centrifugation as in step 2. Wash the glands 3× with 1 mL of Reddy's medium as in 4.

10. Allow the glands to recover in 1 mL of Reddy's medium for 2 h at 27°C.
11. Heat shock the glands by placing in a 41°C water bath for 1 h.
12. Allow the glands to recover at 27°C for a further 2 h.
13. Assay the salivary glands for reporter gene activity.

40.3.3 Metabolic labelling of cultured salivary glands

1. Dissect 10 pairs of adult salivary glands into 50 μL *Aedes* saline on a microscope slide (with a single concave depression) and maintain on ice. Dissecting into *Aedes* saline serves to rinse the glands prior to culture. Transfer into 50 μL Reddy's medium in a 96 well microtitre plate. Seal the plate with Parafilm to prevent evaporation.
2. Maintain the glands in culture for 24–48 h at 27°C.
3. Remove the Reddy's medium using a finely drawn Pasteur pipette and rinse the glands with 50 μL Reddy's medium minus cysteine and methionine.
4. Cover the glands with 50 μL of fresh Reddy's -cys, -met and 1 μL (10 μCi) ^{35}S translabel containing ^{35}S-labelled cysteine and methionine.
5. Incubate the glands at 27°C for 5–8 h.
6. Remove the supernatant using a finely drawn Pasteur pipette and reserve for analysis. Wash the glands with 50 μL nonlabelled Reddy's medium and resuspend in 50 μL of the same.
7. Transfer the glands, using a dissecting needle, to 50 μL of Reddy's medium in a Kontes tube and homogenize using a Kontes pestle. Freeze–thaw the glands twice by alternately placing on dry ice for 5 min and then at 37°C for 5 min. Analyse gland homogenates and culture supernatants using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).
8. After electrophoresis, incubate the gel for 20 min with gentle shaking in a methanol (40:10) solution. Repeat this incubation twice with fresh solution to fix the gel and remove unincorporated label.
9. Incubate the gel with Enhance™ for 20 min and vacuum dry for 1 h 15 min at 80°C.
10. Expose the gel to X-ray film for 7–20 h at RT.

NOTES

1. Using 40× magnification, the basal lamina is visible surrounding the cells of the salivary gland. For adult *Ae. aegypti* glands, the basal lamina is effectively removed by a 45 min treatment in 0.2% elastase. This was determined empirically by observing the salivary glands in 0.2% elastase for varying time periods. Conditions of treatment should be similarly determined for salivary glands of other species. Effective removal of the basal lamina exposes the cells of the glands, and can be distinguished by the appearance of an irregular, crenulated outline of the individual lobes. Excessive treatment with elastase results in dissociation of the epithelial cells from the salivary duct. Following removal of the basal lamina, the gland structure is extremely fragile and unnecessary manipulation of the glands should be avoided.

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CHAPTER FORTY ONE

Culture and analysis of the insect fat body

Alexander S. Raikhel, Kirk W. Deitsch and Thomas W. Sappington

41.1 INTRODUCTION

In insects, the fat body is the centre of intermediate metabolism: it is involved in the metabolism and storage of carbohydrates, lipids, and nitrogenous compounds. Moreover, it is a powerful secretory organ responsible for the production of virtually all haemolymph proteins, including most immune defense factors. The fat body is capable of extremely high levels of protein synthesis and secretion, as exemplified by the massive production of yolk protein precursors during vitellogenesis in female insects. Throughout the life cycle, fat body functions are developmentally or hormonally regulated, accommodating the needs of the developing, growing, metamorphosing, or reproducing insect (Wyatt, 1980; Keeley, 1985; Kanost *et al.*, 1990; Raikhel, 1992).

The fat body is composed of lobes or sheets of tissue that are distributed throughout the insect. Although present in the head, thorax, and even the legs, the majority of this tissue is located in the abdomen, adhering to the epidermis and surrounding the midgut. The fat body's extensive surface area, exposed to the haemolymph, facilitates rapid metabolite exchange and efficient protein secretion (Dean *et al.*, 1985; Raikhel, 1992).

The cellular composition of the fat body varies considerably among insects. Trophocytes, or adipocytes, make up the majority of this tissue and are responsible for most metabolic functions. In some orders of insects, the fat body is composed entirely of trophocytes; in most insects, however, additional specialized cell types are present. Oenocytes, a far less numerous cell type, are located at the surface of fat body lobes in Diptera and Coleoptera. It is thought that these cells may be involved in the metabolism of ecdysteroids (Romer *et al.*, 1975). The colour of aquatic larvae of the Simuliidae is determined by chromatocytes, fat body cells lying beneath the transparent epidermis. Mycetocytes, another specialized fat body cell type found in blood- or plant-feeding bugs (Hemiptera), cockroaches (Orthoptera), and insects from several other orders, harbour symbiotic intracellular microorganisms. These cells are either scattered singly throughout the fat body tissue, as in Hemiptera and termites, or are located in groups near the centre of fat body lobes, as in cockroaches. The urocytes, occurring principally in cockroaches, are fat body cells that store nitrogen metabolism products called urates (Dean *et al.*, 1985; Keeley, 1985).

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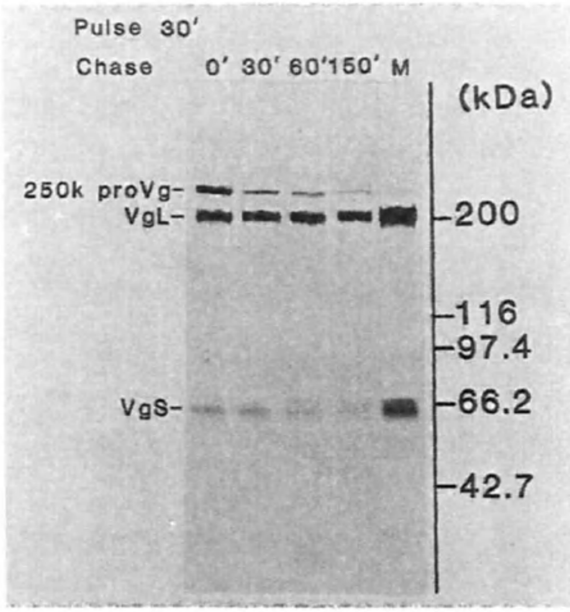
Because of the fat body's physiological significance and the ease of *in vitro* manipulations, this insect tissue was one of the first to be cultured for both vector and non-vector insects. Among vector arthropods, most research involving fat body culturing has been performed using mosquitoes, with these techniques finding only limited employment in other species (Taylor *et al.*, 1991; Valle *et al.*, 1992). *In vitro* culturing of the *Aedes aegypti* fat body has established this tissue as the site of vitellogenin (Vg) synthesis (Hagedorn and Judson, 1972) and 20-hydroxyecdysone (20E) as the hormone regulating Vg synthesis (reviewed in Hagedorn, 1985; Raikhel, 1992; Dhadialla and Raikhel, 1994).

Fat body culturing has been widely used to analyse proteins produced by this tissue (Kunkel and Nordin, 1985; Raikhel and Dhadialla, 1992; Raikhel, 1992). In the mosquito, this technique has been successfully adapted for the production of fat body proteins to study their properties (Raikhel and Bose, 1988; Dhadialla and Raikhel, 1990; Hays and Raikhel, 1990) and their uptake by the ovary through receptor-mediated endocytosis (Koller *et al.*, 1989; Koller and Raikhel, 1991; Dhadialla and Raikhel, 1991; Dhadialla *et al.*, 1992; Sappington *et al.*, 1995).

Fat body culturing has proved a key technique in elucidating the biosynthetic pathway of mosquito Vg (Dhadialla and Raikhel, 1990). *In vitro* pulse-chase labelling experiments utilizing [^{35}S]-methionine have revealed the intermediate products in Vg biosynthesis: a Vg precursor (M_r = 250 000) exists as a distinct intermediate and is cleaved into two polypeptides with M_rs of 190 000 and 62 000, which mature to 200 kDa and 66 kDa large and small Vg subunits, respectively (Figure 41.1A). Tunicamycin, an inhibitor of N-linked glycosylation, has been used in *in vitro* labelling experiments to show that the glycosylation of Vg occurs co-translationally (Figure 41.1B). Additionally, the co- and post-translational modifications of the pro-Vg and Vg subunits, glycosylation, phosphorylation, and sulfation, have been analysed by *in vitro* incorporation of radiolabelled precursors. Moreover, the use of monensin, an ionophore affecting the function of the Golgi complex, in labelling experiments has demonstrated that the maturation of both Vg subunits occurs as one of the last steps prior to Vg secretion. Prevention of sulfation by this drug established that this protein modification takes place during the final maturation of Vg subunits (Dhadialla and Raikhel, 1990).

Fat body culturing has been instrumental in the discovery and analysis of two novel yolk protein precursors, vitellogenic carboxypeptidase (VCP) and vitellogenic thiol protease, which are produced by the *Ae. aegypti* fat body and internalized by oocytes (Hays and Raikhel 1990; Cho *et al.*, 1991).

Deitsch *et al.* (1995) have extended the research on 20E-control of mosquito gene expression through the utilization of fat body culture and analysis of two yolk protein genes, Vg and VCP. The effect of cycloheximide (CHX), an inhibitor of protein synthesis, on Vg and VCP gene expression was tested *in vitro*: physiological doses of 20E initiated expression of both yolk protein genes in previtelogenic fat bodies, but the effect of 20E was inhibited by pretreatment of cultured fat bodies with CHX (Figure 41.2). Thus, the authors concluded that 20E action on the mosquito yolk protein genes, Vg and VCP, is indirect and requires one or more other gene products, and that the action of 20E is mediated by a regulatory cascade similar to that of *Drosophila*. The use of mosquito fat body culture has also demonstrated that 20E negatively regulates the translation of lysosomal enzyme mRNA at the end of the vitellogenic cycle (W.L. Cho and A.S. Raikhel, unpublished data). In the future, the application of fat body culturing techniques in studies of regulation of gene expression will broaden as more fat body-specific genes are cloned.



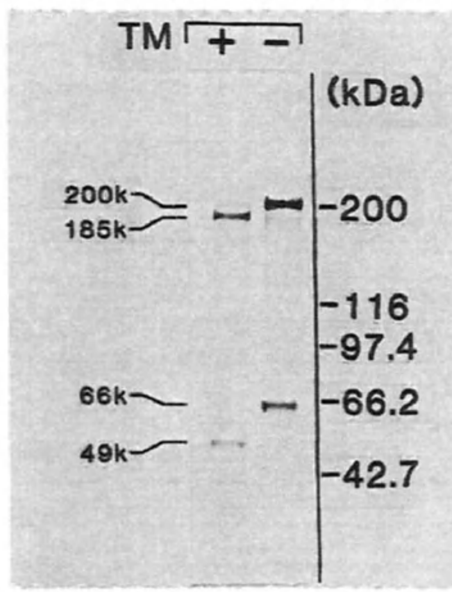


Figure 41.1 Analysis of protein processing (mosquito vitellogenin) using fat body culture. (A) Time course of ^{35}S-methionine labelling of the Vg precursor. Fat bodies dissected from previtellogenic female mosquitoes were incubated for 6 h in culture medium containing 10^{-5} M 20E. The fat bodies were then pulse-labelled by incubating them for 30 min in the methionine-culture medium supplemented with ^{35}S-methionine, and chased for the indicated times in complete culture medium. Fat bodies were homogenized and proteins immunoprecipitated by a mixture of monoclonal antibodies against the large and small Vg subunits. The immunoprecipitates were resolved by SDS-PAGE on a 5–10% gradient gel. Proteins secreted to the 150-min medium (M) were analysed by immunoprecipitation. 250k proVg, vitellogenin precursor; VgL, large Vg subunit; VgS, small Vg subunit. (B) Inhibition of Vg processing by tunicamycin, a drug affecting *N*-linked glycosylation. Vitellogenic fat bodies (18–22 h post-blood meal) were preincubated in mosquito Ringer's solution with 25 mM TES, pH 7.5, for 1 h. The fat bodies were pulse-labelled for 1 h in incomplete media with ^{35}S-methionine. Preincubation and labelling were done in the continued presence or absence of tunicamycin. Vg was immunoprecipitated from fat body extracts as in (A). Results show that processing of both subunits is inhibited in the presence of tunicamicyn (TM+). Additional labelling with D-[2-^{3}H]mannose and ^{32}P-orthophosphate demonstrated that tunicamycin inhibited both glycosylation and phosphorylation of Vg subunits (not shown). TM, tunicamycin; 200k, fully processed large Vg subunit; 185k, large Vg subunit inhibited by TM; 66k, fully processed small Vg subunit; 49k, small Vg subunit inhibited by TM. The standards in order of decreasing M_r are: myosin, β-galactosidase, phosphorylase b, bovine serum albumin, and ovalbumin (Bio-Rad). (From Dhadialla and Raikhel (1990), with permission.)

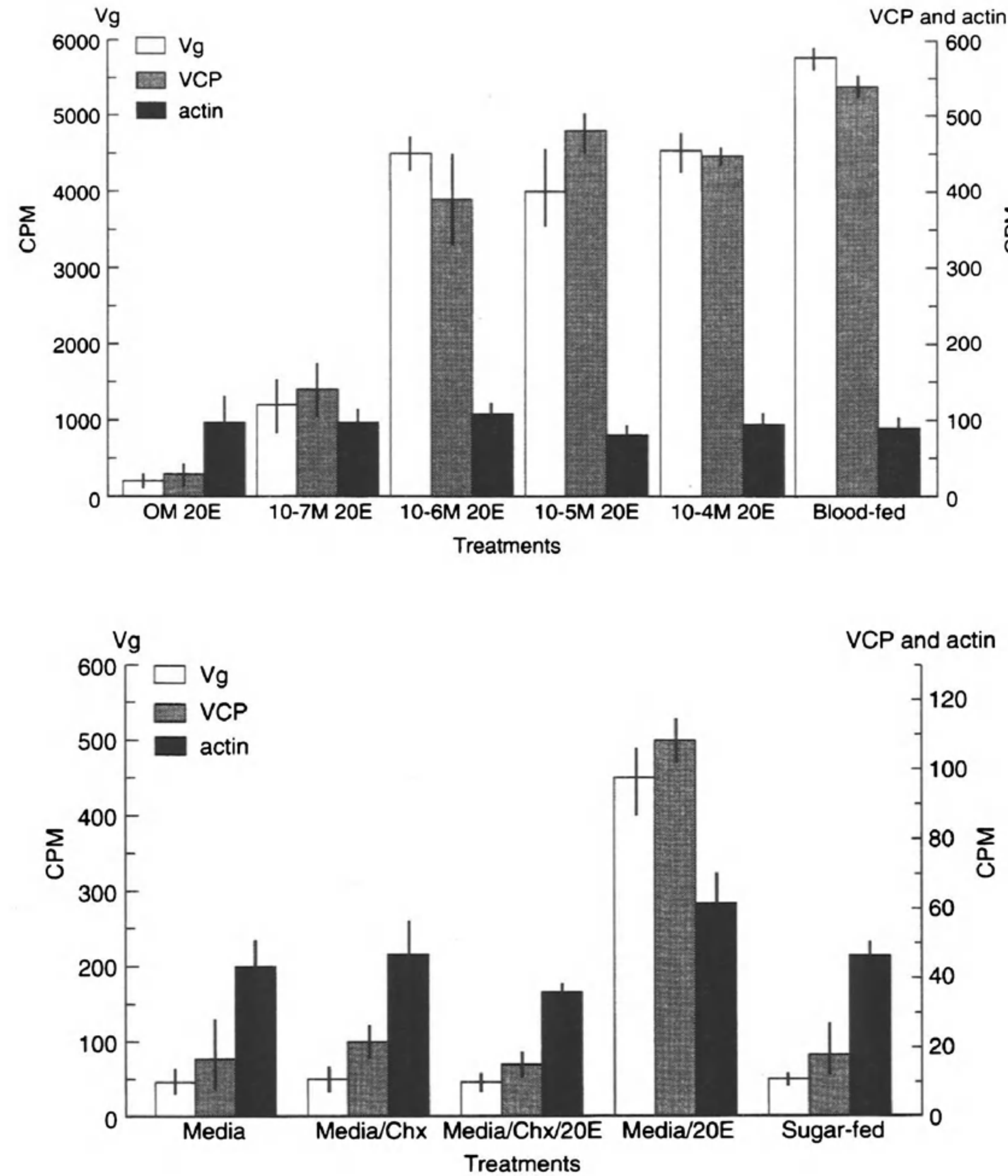


Figure 41.2 Analysis of hormonal induction of gene expression using fat body culture. (A) Effect of 20-hydroxyecdysone (20E) concentration on Vg, vitellogenic carboxypeptidase (VCP), and actin mRNA production in cultured mosquito fat bodies. Previtellogenic fat bodies were cultured for 6 h in the absence or presence of 20E at concentrations indicated. The medium was changed every 2 h. Fat bodies from females 6 h post-blood meal (blood-fed) were used to determine the levels of these mRNAs after normal stimulation of vitellogenesis. (B) Effect of cycloheximide (ChX) on 20E-induction of Vg and VCP gene expression in cultured fat bodies. Previtellogenic fat bodies were preincubated in the culture medium in the presence or absence of 10^{-4} M ChX for 1 h, then cultured for 6 h in media containing different combinations of ChX and 20E (10^{-5} M) as indicated. Fat bodies from previtellogenic, 3-day-old (sugar-fed) females were used as a control. Total RNA was extracted and levels of Vg, VCP, and actin mRNA measured by slot blot and hybridization to the respective radiolabelled cDNA probes. Hybridization was quantified using a scintillation counter. Vertical lines in each bar indicate the standard error of the mean of three repetitions. (From Deitsch *et al.*, 1995, with permission.)

41.2 MATERIALS

41.2.1 Metabolic radiolabelling and purification of *Aedes* Vg from fat body culture

1. Complete/incomplete culture medium: ('incomplete' lacks methionine which is added separately as [^{35}S]-methionine.) All solutions are made in ddH_2O (Milli-Q or equivalent). This medium was modified from that of Bohm *et al.* (1978) by Koller *et al.* (1989) by raising the concentrations of leucine from 1 mM to 5 mM and NaCl from 25 mM to 140 mM, and by eliminating polyvinylpyrolidine-40 and nucleotides (Note 1). Instructions for preparation of the medium are in Chapter 40. The medium can be aliquoted and stored at –80°C for 1 year. Just prior to use, add 2 μL aprotinin stock (5 mg/mL in ddH_2O) per mL of culture medium (Note 2).
2. APS (*Aedes* physiological saline): 150 mM NaCl, 4 mM KCl, 0.1 mM $NaHCO_3$, 0.6 mM $MgCl_2$, 25 mM HEPES buffer. Adjust pH to 7.0 at 27°C with NaOH. Add $CaCl_2$ to 1.7 mM. Filter (0.45 μm pore size). Store at 4°C. Stable 1 month.
3. Absolute ethanol.
4. 70% ethanol: dilute with ddH_2O.
5. [^{35}S]-methionine: 10 mCi/mL.
6. IEX (ion-exchange) starting buffer: 20 mM Tris-HCl, 150 mM NaCl, 0.02% sodium azide, pH 7.5 at 4°C.
7. IEX middle buffer: 20 mM Tris-HCl, 250 mM NaCl, 0.02% sodium azide, pH 7.5 at 4°C.
8. IEX elution buffer: 20 mM Tris-HCl, 400 mM NaCl, 0.02% sodium azide, pH 7.5 at 4°C.
9. Microtitre plate (e.g., Serocluster U Vinyl Plate, Costar).
10. Protease inhibitors:
    a. PI mix: Preparation of stock containing antipain, leupeptin, aprotinin, chymostatin:
    i. Dissolve 1 mg each antipain and leupeptin in 100 μL ddH_2O.
    ii. Dissolve 2.5 mg aprotinin in 100 μL ddH_2O.
    iii. Dissolve 1 mg chymostatin in 250 μL DMSO.
    iv. Combine all: 100 μL leupeptin + antipain
    100 μL aprotinin
    250 μL chymostatin
    50 μL ddH_2O

    Final solution will consist of 2 mg/mL leupeptin, antipain, and chymostatin and 5mg/mL aprotinin. Use 2 μL/mL. Store at –20°C. Stable for 1 month.
    b. AEBSF: 200 mM in pH 7.0 buffer. Use 5 μL/mL. Store at 4°C. Stable for 6 months.
    c. EDTA: 0.5 M in pH 8.5 buffer. Use 25 μL/mL. Store at 4°C. Stable for 6 months.
    d. ACA/BAM: 200 mM E-amino *n*-caproic acid, 40 mM benzamidine in ddH_2O. Use 25 μL/mL. Store at 4°C. Stable for 1 month.
    e. Pepstatin A: 1 mM pepstatin A in methanol. Use 2 μL/mL. Store at 4°C. Stable for 1 week.
11. Incubation buffer: 200 mM NaCl, 4 mM KCl, 1.8 mM $NaHCO_3$, 0.6 mM $MgCl_2.H_2O$, 18.8 mM TES free acid, 6.25 mM TES sodium salt. Adjust pH to 7.2

at 4°C with NaOH. Add $CaCl_2$ to 1.7 mM. Filter (45 μm pore size). Store at 4°C, make fresh monthly.

12. Desalting (gel exclusion) column: 2–3 mL dead volume.
13. IEX (anion exchange) column: DEAE-Sepharose CL-6B for conventional liquid chromatography, or HR 5/5 MonoQ (Pharmacia) for HPLC, or equivalents.
14. Centricon-30 concentrator (Amicon, or equivalent).
15. Two pairs of super-fine point forceps: e.g. Dumont Biology Grade Tweezers, 110 mm, points 0.05 × 0.01 mm width × thickness.
16. Vannas Micro Scissors, straight or angled, 80 mm (3′).

41.2.2 Pulse–chase analysis of *Aedes* Vg synthesis in fat body culture

1. Incomplete culture medium: (Section 41.2.1a).
2. Complete culture medium: Same as incomplete culture medium, but containing 2 mM L-methionine.
3. [^{35}S]-methionine: 10 mCi/mL.
4. APS: (Section 41.2.1).
5. Absolute ethanol.
6. 70% ethanol: dilute in ddH_2O.
7. Protease inhibitors: (Section 41.2.1)
8. Microtitre plate: (Section 41.2.1).
9. Homogenization buffer: 50 mM Tris-HCl, 50 mM NaCl, 0.2% Triton X-100, 0.02% sodium azide, pH 7.6 at 4°C.
10. 2× RIPA buffer: 2% Triton-X-100, 2% sodium deoxycholate (*do not breathe dust*), 0.2% sodium dodecyl sulfate, 0.3 M NaCl, 40 mM Tris. Adjust pH to 7.4 with HCl. Dilute 1:1 with water or sample. Store at RT. Stable 2 months.
11. Protein-A sepharose (50% suspension):
    a. To 0.1 g dry Protein-A sepharose, add 1200 μL 1× RIPA buffer containing all protease inhibitors.
    b. Shake 1 h at 4°C.
    c. Spin 1 min at highest speed on bench-top centrifuge and discard supernatant.
    d. Repeat a–c 2×.
    e. Add volume of 1× RIPA (with all protease inhibitors) equal to the Protein-A Sepharose packed volume (= 50% suspension, packed-vol/vol).
12. 20 mM Tris. Adjust pH to 7.5 with HCl.
13. 2× SDS-PAGE sample buffer: 100 mM Tris Base, 4% SDS, 12% glycerol, ~0.03 mg/mL bromophenol blue. Adjust pH to 6.8 with HCl, then add DTT (dithiothreitol) to 150 mM. Aliquot and freeze as soon as possible. Stable at –20°C. Do not reuse after thawing.
14. Sigmacote (or equivalent): for siliconizing surfaces.
15. Spin filter tube: puncture small hole in bottom of a 500 μL microcentrifuge tube with a syringe needle. Use large end of a glass Pasteur pipette to cut a disc from a glass-fibre filter (e.g. Whatman GF/C), then cut disc in half. Push half-disc to bottom of microcentrifuge tube to cover the hole. Dispense 200 μL of Sigmacote (or equivalent) into the tube and let stand overnight. Remove Sigmacote and let dry completely.

16. RIPA/BSA/MET buffer: 2% bovine serum albumin, 2% nonradioactive methionine, in 1× RIPA buffer.
17. Saturated TCA: Trichloroacetic acid-saturated H_2O at 4°C.
18. 20% TCA: 20% of saturated TCA.
19. 5% TCA: 5% of saturated TCA.
20. TCA/MET: 5% TCA with 1% nonradioactive methionine.
21. BSA stock: 10 mg/mL bovine serum albumin in ddH_2O.
22. GF/C Whatman filters.
23. Filtration manifold (e.g. Millipore model 1250 or equivalent).
24. EnHance: 67% glacial acetic acid, 28% 2-ethoxyehtanol (Kodak 1697), 50 mL 2-methylnapthalene (practical grade) (Kodak P 2416), 5 g/L PPO. Melt methylnapthalene in 60°C water bath and add to acetic acid and ethoxyethanol. Add PPO last. *Do all work with EnHance in a hood.*
25. Coomassie Brilliant Blue Stain: 50% methanol, 10.5% glacial acetic acid, 2.5 g/L Coomassie Brilliant Blue R-250 in ddH_2O. Dissolve Coomassie blue in methanol then add acetic acid and water.
26. Formaldehyde fixer: 1% formaldehyde (from standard 37% stock), 7% glacial acetic acid, 25% methanol in ddH_2O.

41.3 METHOD

41.3.1 Metabolic radiolabelling and purification of *Aedes* Vg from fat body culture

The following method has been adapted from Koller *et al.* (1989) and Dhadialla and Raikhel (1991).

1. Prepare a microtitre plate: Fill wells with 70% ethanol and let stand 10 min. Rinse wells with absolute ethanol and let dry.
2. Rinse all instruments (forceps, scissors, dissecting slides) with 70% ethanol to reduce any bacterial contamination. Let air-dry thoroughly.
3. Add 30 μL of $[^{35}S]$-methionine to 2 mL incomplete culture medium with aprotinin. Dispense 35–37 μL of this medium per well into ~55 wells of the microtitre plate. *Do all work with $[^{35}S]$-methionine in a hood.*
4. Anaesthetize the mosquitoes with ether or CO_2. Surface sterilize the anaesthetized insects by briefly submerging them in 70% ethanol before dissection. Allow them to air-dry on a tissue before beginning the dissection.
5. Place the mosquito on its dorsum in RT APS in the well of a dissecting slide under a dissecting microscope. Grasp the thorax firmly, but without crushing, with one pair of forceps. With another pair of forceps, tightly grasp the penultimate abdominal segment and pull. The ovaries and Malpighian tubules usually pull out with the abdominal tip and can be discarded (Figure 42.3). If you are working with previtellogenic (i.e. nonblood-fed) mosquitoes, pull out the midgut at the same time. Using microscissors, cut along one side of the cuticle from the open abdominal tip to the thorax, either dorsally through tergites or ventrally through sternites (be careful not to puncture the blood-filled midgut (Note 3). Cut the abdomen from the thorax, removing and discarding the head

and thorax. Push out the midgut by gently squeezing the abdomen and discard it. The abdomen should open up, or 'butterfly', with the cuticle remaining dry on the surface of the saline solution and the fat body tissue extending down into the liquid (Figure 41.3).

6. Transfer the fat body to the culture medium, four fat bodies/well: grasp the isolated abdomen by one edge with the forceps and lift it from the slide. Once removed from the dissecting saline, the abdomen will likely fold around the tip of the forceps. However, when placed in the culture medium, it should immediately open up and float on the surface with the cuticle on top and the fat body tissue extending into the media (Figure 41.3). It is important that the isolated tissue be in this configuration. If the abdomen sinks or curls up, the tracheal system will not be exposed to the atmosphere and the underlying fat body will no longer be properly oxygenated (Note 4). Fat bodies that cannot be made to float properly should be discarded. Keep the microtitre plate covered with a lid or

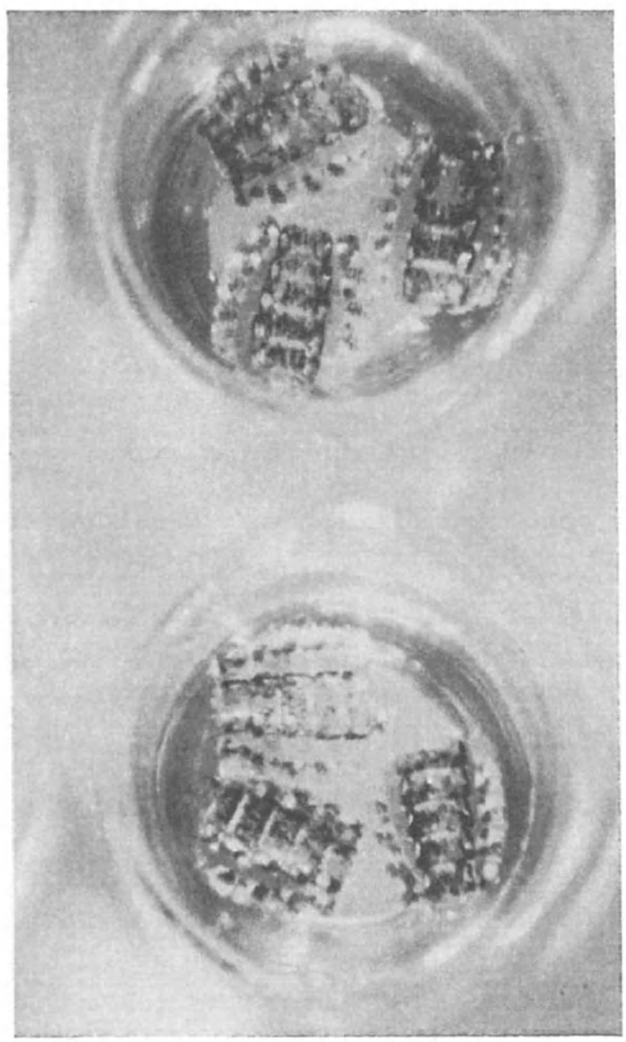


Figure 41.3 Cultured mosquito fat body. Abdominal wall with the adhering fat body (referred to elsewhere simply as fat body) was dissected as described in the text. Three abdominal preparations are placed in a well containing 100 μL of culture medium. Culture medium is replaced every 2 h. Note that the abdominal walls are spread and floating on the surface of the medium. (Photo by T.S. Dhadialla.)

Parafilm, and place the entire plate into a closed container with wet paper towels to maintain a humid atmosphere.

7. Allow the fat bodies to incubate for about 2 h at 27°C (Note 5).
8. In each of two 1.5 mL microfuge tubes, dispense 400 μL IEX starting buffer and all protease inhibitors, assuming a final volume of 1.5 mL in each tube. Keep these tubes on ice.
9. With two people working, it will take at least 2 h to do the dissections (200–220 mosquitoes). Thus, the medium containing the first cohort of fat bodies will be ready to harvest before or soon after the last cohort has been dissected. Collect incubation medium every 30 min from wells in the order of fat body introduction (~12–15 wells per 30 min sample), and dispense into the microfuge tubes prepared in the previous step. Keep the samples on ice. The medium will contain [^{35}S]-Vg synthesized and secreted by the fat bodies.
10. Centrifuge the samples at 14 t000 **g** for 10 min at 4°C. Transfer the supernatants to new tubes and discard the pellet as radioactive waste.
11. Flush the desalting column with IEX starting buffer. Measure the volume and introduce the samples (supernatants from previous step) to the top of the column and allow this solution to enter the gel matrix. If the volume of the combined samples is less than the dead volume of the column, add the volume of IEX starting buffer necessary to make up the difference and allow it to enter the matrix. Add a volume of IEX starting buffer, equivalent to or slightly more than the loaded sample volume, and collect the fluid leaving the column. This fluid will contain the [^{35}S]-Vg sample in IEX starting buffer.
12. Introduce the sample from the desalting column to the anion exchange column equilibrated in IEX starting buffer (150 mM NaCl). Discard the unbound fraction as radioactive waste. Flush the column with IEX middle buffer (250 mM NaCl) and discard the eluted fraction. Elute and collect purified [^{35}S]-Vg with IEX elution buffer (400 mM NaCl).
13. The [^{35}S]-Vg must be concentrated and exchanged into incubation buffer: Add a small volume of ddH$_2$O to a Centricon-30 concentrator and centrifuge at 2000 **g** for 10 min to wet the filter. Discard the water and load up to 3 mL of Vg sample. Centrifuge at 6000 **g** for ~40 min (do not let the filter spin dry). Continue adding and spinning the sample in this way until it has been concentrated to about 100–200 μL. Add 1 mL incubation buffer and concentrate as before to about 100 μL. Transfer this solution to a microfuge tube and rinse the filter 3× each with ~70 μL incubation buffer. Combine the rinses with the concentrate. Measure the protein concentration, aliquot, and store at –80°C. The frozen Vg is stable indefinitely but should not be thawed more than twice.

41.3.2 Pulse–chase analysis of *Aedes* Vg synthesis in fat body culture

The following protocol is adapted from Raikhel and Bose (1988), Dhadialla and Raikhel (1990) and Hays and Raikhel (1990).

1. For each treatment, incubate fat bodies of 5 mosquitoes 18–22 h post-blood meal in 100 μL incomplete culture medium containing [^{35}S]-methionine as described in Section 41.3.1, steps 1–6 (Note 6). Time of incubation, or pulse time, in the medium containing radiolabelled methionine can be as short as 5 min or as long

as 2 h depending on the purpose of the experiment. As a beginning guide, pulse for 30 min.

2. 'Chase' the [^{35}S]-labelled Vg and Vg precursors: Rinse the fat bodies 3× in APS or complete culture medium, then incubate them in complete culture medium containing no radiolabelled methionine. Chase time can be varied depending on the purpose of the experiment. As a beginning guide, set up five chase-treatments of 0, 15, 30, 60, and 120 min.
3. Harvest the chase medium and the fat bodies separately. These can be stored in liquid N_2.
4. Add 200 μL of homogenization buffer (containing all protease inhibitors) for each five fat bodies and homogenize by sonication (this solution will be referred to as fat body extract). Add an equal volume (100 μL) of homogenization buffer (containing all protease inhibitors) to the harvested chase medium (this solution will be referred to as fat body secretion). Centrifuge the fat body extracts and secretions at 14 000 **g** for 20 min to remove cell debris. Transfer supernatants to new tubes and discard the pellets (Note 7).

At this point, fat body extracts or secretions can be immunoprecipitated to isolate a specific radiolabelled protein (Section 41.3.2a), or total radiolabelled proteins can be sampled by precipitating with trichloroacetic acid (TCA) (Section 41.3.2b).

(a) Immunoprecipitation of Aedes *Vg*

The following procedure requires polyclonal or monoclonal antibodies to the protein of interest, in this case *Ae. aegypti* vitellogenin (Note 8).

1. Add 45 μL of antiserum (Note 9). Incubate on ice for 2 h.
2. Add 40 μL of Protein-A Sepharose suspension. Incubate overnight at 4°C on a shaker.
3. Centrifuge at highest speed in bench-top centrifuge for 1 min. Remove RIPA buffer (carefully so as not to lose any Sepharose beads). Resuspend in 200 μL 1× RIPA buffer.
4. Repeat previous step 3x, except after the final pelleting, do not resuspend in RIPA buffer.

At this point the immunoprecipitated samples may be processed for either filter radioimmunoassay (Section 41.3.2a(i)) or SDS-PAGE and fluorography (Section 41.3.2a(ii)) both of which will be described, although details of SDS-PAGE will not be given. For excellent descriptions of SDS-PAGE methodology, we refer the reader to Smith (1984) and Walker (1984).

(i) Filter radioimmunoassay of immunoprecipitate

1. Resuspend the immunoprecipitated pellet from step 8 (Section 41.3.2a) above in 200 μL RIPA/BSA/MET buffer.
2. Prepare 1 GF/C Whatman filter for each sample: Block binding sites on each filter with Sigmacote at 200 μL per filter. Let dry completely. Under vacuum in a filtration manifold, wash the filters with 2.5 mL RIPA/BSA/MET.
3. Apply the total volume of washed immunoprecipitate to the centre of the filter while under vacuum. Wash the tube 3× with 200 μL RIPA/BSA/MET and apply each wash to the filter under vacuum.
4. Wash the filters 4× with 2.5 mL RIPA/BSA/MET. Drip the buffer carefully on the Sepharose beads to disturb them as little as possible.

5. Place each filter in an open scintillation vial and dry under vacuum (~15 min). Add 5 mL scintillation fluid. Do not vortex. Let stand until the filter becomes clear. Take scintillation counts.

(ii) SDS-PAGE and fluorography of immunoprecipitate

1. Resuspend pellet from step 8 (Section 41.3.2a) above in 200 μL 20 mM Tris pH 7.5. Centrifuge at top speed in bench-top centrifuge for 1 min. Discard supernatant.
2. Add ~2× bead volume (~40 μL) of 2× SDS sample buffer. Boil for 7 min to release the protein from the beads.
3. Transfer all contents to a spin filter tube. Place spin filter tube inside a 1.5 mL microfuge tube. Spin at 2000 RPM in a bench-top centrifuge for 2 min.
4. Wash beads 1× with ~35 μL 2× SDS sample buffer and spin through. Discard the filter with the trapped Sepharose beads.
5. Count 5–10 μL of the filtrate in a scintillation counter.
6. Load up to 80 μL/gel lane for SDS-PAGE.
7. After SDS-PAGE, stain and fix the gel in 10% Coomassie stain in formaldehyde fixer for 30 min – overnight.
8. Discard stain-fixer solution in radioactive waste container.
9. Soak gel in EnHance on shaker for at least 30 min.
10. Discard EnHance into a radioactive waste container.
11. Briefly rinse the gel once with ddH_2O, then soak it in ddH_2O for 30–45 min on a shaker. Discard both rinses in a radioactive waste container.
12. Dry the gel: Place the gel on Whatman 3MM filter paper and cover with plastic wrap. Dry the gel for 1 h at 80°C under vacuum. Cool the gel without heat under vacuum for 20–30 min. Break the vacuum and remove the plastic wrap. The gel is now ready for exposing to film.

(b) Precipitation and assay of total radiolabelled protein

1. Dispense a 10 μL subsample of fat body extract or secretion into a microfuge tube. Add 5 μL of BSA stock and 50 μL of ddH_2O and vortex. Add 65 μL of 20% saturated TCA, vortex, and let stand on ice for 1 h.
2. Centrifuge 1 min at 12 000 g and discard supernatant as radioactive waste. Wash the pellet 2× with 5% saturated TCA using a pipette tip to grind it.
3. Suspend the pellet in 5% saturated TCA + 1% methionine. Add 25 μL 1 M NaOH and vortex. Add 25 μL 1 M acetic acid and vortex.
4. Add 1 mL aqueous scintillation fluid and vortex 15 s.
5. Place the microfuge tube in a mini scintillation vial and place that in a standard scintillation vial. Measure counts per min in a liquid scintillation counter (Note 13).

NOTES

1. The medium for *Ae. aegypti* organ culture has evolved for almost three decades from the media reported by Reddy and Wyatt (1967) who cultured *Cecropia* fat bodies, and from that of Hagedorn *et al.* (1973), who first adapted the *Cecropia* medium for use with *Ae. aegypti*. Koller *et al.* (1989) and Dhadialla and Raikhel (1990) optimized the culture medium further as indicated in Section 41.2.1.

Tissues from hematophagous insects generally require higher NaCl and lower KCl concentrations in the medium for optimal metabolism than do those of other insects (Mullins, 1985). Because of this feature, culture media originally developed for mammalian cell lines have been adapted most successfully for culturing mosquito cell lines (Kurtii and Munderloh, 1984; Fallon and Stollar, 1987; Fallon, 1989). Borovsky *et al.* (1994) reported using a commercially available culture medium, 'TC 199' (Gibco), a formulation originally developed for nutritional studies of chick embryo fibroblasts, for culturing *Ae. aegypti* fat bodies, ovaries, guts, and corpora allata. Valle *et al.* (1992) used Eagle Minimal Essential Medium (Gibco), originally developed for mammalian cell lines, to culture *Rhodnius* fat bodies. Wherever possible, we recommend using culture media and buffers that have been adjusted to the requirements of the insect of interest. The rate of total protein synthesis is a good criterion for evaluating culture media.

2. Aprotinin is a protease inhibitor that does not cross the cell membrane. Thus, it helps prevent degradation of secreted protein without affecting the active cells.
3. If a blood-filled midgut is punctured during dissection, that mosquito should be discarded and the dissection saline on the slide replaced with fresh APS. Release of midgut contents exposes the fat body tissue to gut digestive enzymes which can be detrimental to fat body survival.
4. Maintaining a supply of oxygen to the cultured organs is of extreme importance for keeping the tissue alive and metabolically active during the period of culture. Several methods have been used to maintain adequate oxygenation of cultured organs, including keeping the tissue in an environment rich in oxygen (Riddiford *et al.*, 1979), adding oxygen-carrying perfluorochemical emulsion to the media (Noriega and Wells, 1992), and keeping the tracheal system intact and open to allow the tissue to obtain oxygen directly from the atmosphere (Kaatz *et al.*, 1985). The fat body of small insects, such as mosquitoes, is particularly well-suited for the latter approach. Because of its location lining the abdominal wall, the fat body can be cultured by isolating the abdomen of the insect as described in Section 41.3.1, step 5. Cultured fat bodies remain metabolically active for many hours without considerable losses in levels of protein synthesis.
5. In experiments calling for longer fat body incubation, the culture medium is generally changed every 2 h, unless the experimental design dictates more frequent media changes. This ensures a fresh supply of amino acids and other nutrients to the tissue throughout the experiment.
6. [^{35}S]-cysteine and a [^{14}C]-amino acid mixture were found to be equally as adequate for pulse-labelling proteins in fat body culture as [^{35}S]-methionine (Dhadialla and Raikhel, 1990).
7. Specific moieties which are added to a synthesized protein during its processing in the fat body can be monitored by pulse–chase experiments as well. An appropriate radiolabelled precursor is introduced to the fat body culture medium in place of [^{35}S]-methionine. The following moieties have been analysed in this way for *Ae. aegypti* Vg (Dhadialla and Raikhel, 1990) and vitellogenic carboxypeptidase (VCP; formerly 53KP) (Hays and Raikhel, 1990):
Carbohydrates: Incubate the fat bodies in APS containing 0.1 mCi/mL of D-[2-^{3}H]-mannose. Chase in complete culture medium containing 50 μM sodium pyruvate.
Phosphates: Incubate the fat bodies in complete culture medium but with K_2HPO_4 replaced by 2 mM K_2HCO_3, and containing [^{32}P]-orthophosphoric acid

(40 μCi/mL), 5 mM EGTA, 50 μM dibutyryl cAMP, and 50 μM 3-isobutyl-1-methyl xanthine.

Sulfates: Incubate the fat bodies in complete culture medium that has been modified as follows: eliminate L-tyrosine, reduce both L-methionine and L-cysteine to 40 μM, eliminate streptomycin sulfate, and add sodium [^{35}S]-sulfate (2 mCi/mL).

The proteins in the fat bodies and chase media from these experiments can then be processed for immunoprecipitation (Section 41.3.2a).

8. The procedure for immunoprecipitation takes advantage of the affinity of immunoglobulins for Protein-A to sequester antibody-antigen complexes from solution. The Protein-A is immobilized on Sepharose beads allowing isolation of attached complexes by centrifugation. Alternatively, Pansorbin (*Staphylococcus aureus* cells with Protein-A attached) can be used as the precipitating agent. However, preincubation of the sample with Pansorbin is necessary to reduce nonspecific interactions, and Hays and Raikhel (1990) found that this reduced sensitivity of the assay compared to immunoprecipitation with Protein-A Sepharose.
9. The volume of antiserum may have to be adjusted depending on the concentration of the antibody and its affinity for the antigen.
10. In several of the notes that follow, methods are described for testing the effects of various drugs (tunicamycin, monensin, cycloheximide) on protein synthesis and processing in cultured fat bodies. When using drugs such as this in organ culture, *it is very important to perform preliminary titration experiments to determine the optimal dose*, i.e. the minimal dose that has the desired primary effect without significantly reducing protein synthesis. Titration experiments also should be performed when testing the effects of hormones, such as 20E, on protein synthesis.
11. Glycosylation of proteins can be studied by incubating fat bodies in the presence of the *N*-glycosylation inhibitor tunicamycin, as was done for mosquito Vg (Figure 41.1B) (Dhadialla and Raikhel, 1990) and VCP (Hays and Raikhel, 1990): Preincubate fat bodies for 1 h in uptake Ringer's (150 mM NaCl, 4 mM KCl, 0.1 mM $NaHCO_3$, 0.6 mM $MgCl_2$, 25 mM TES buffer; adjust pH to 7.5 at 27°C with NaOH; add $CaCl_2$ to 3.0 mM) containing the optimal dose of tunicamycin. As a beginning guide for titration, try 0, 0.1, 0.25, and 0.5 mM tunicamycin. Dhadialla and Raikhel (1990) found the optimal dose to be 0.25 mM for *Ae. aegypti* fat bodies. Transfer the fat bodies to incomplete culture medium that has had its pH adjusted to 7.5, and pulse-label with [^{35}S]-methionine in the continued presence of tunicamycin for 15 min. Chase for 30 min in complete culture medium containing the same concentrations of tunicamycin as in the preincubation. Harvest the fat bodies and chase media and process for immunoprecipitation (Section 41.3.2).
12. The Golgi apparatus is the site where several types of protein modifications occur, including *O*-glycosylation with GalNAc, modification of *N*-glycosyl groups, and sulfation of Tyr residues (Creighton, 1993). By adding monensin, an ionophore that inhibits function of the *trans*-Golgi, to fat body culture, one can gain insight into the pathway of protein processing. In mosquito fat body culture experiments, Dhadialla and Raikhel (1990) used monensin to demonstrate that glycosylation and phosporylation of *Ae. aegypti* Vg take place before entering the *trans*-Golgi apparatus and that sulfation occurs in it. Monensin is stored as a 10 mM stock solution in absolute ethanol. Preincubate fat bodies for 30 min in incomplete culture medium, pulse-label 1 h with [^{35}S]-methionine in incom-

plete culture medium, then chase 1 h in complete medium. Monensin should be present in all media at the optimal dose determined by a titration experiment. As a beginning guide for titration, try 0, 10^{-7}, 10^{-6}, and 10^{-5} M concentrations. Dhadialla and Raikhel (1990) determined the optimal dose for *Ae. aegypti* fat bodies to be 10^{-6} M. Harvest the fat bodies and chase media and process for immunoprecipitation (Section 41.3.2).

13. Total radiolabelled proteins from pulse–chase experiments also can be measured by filter radioimmunoassay: Add 100 μg BSA to each fat body extract or secretion, add an equal volume of cold 20% saturated TCA, and precipitate on ice 3 h to overnight. Perform a filter radioimmunoassay as described in Section 41.3.2a(i), except use TCA/MET for the washes, and apply the washes to the filters forcefully and at an angle, creating a vortex to wash the sides of the manifold-well. Alternatively, precipitated radiolabelled proteins can be subjected to SDS-PAGE and fluorography (Section 41.3.2a(ii)).
14. Fat body culture can be used to study gene expression, as was done for the vitellogenic genes encoding mosquito Vg and VCP (Deitsch *et al.*, 1995). Deitsch *et al.* (1995) wanted to determine whether 20E directly stimulates expression of yolk protein genes in the fat body of *Ae. aegypti*, or if its action is indirect, operating through a cascade of genes initially induced by 20E. Answering this question required a series of experiments involving fat body culture:
    a. Optimal 20E concentration was determined by incubating previtellogenic (i.e. nonblood-fed) mosquito fat bodies in complete culture media (Section 41.2.2b) for 6 h in the presence of 0, 10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} M 20E (Figure 41.2A). Total RNA was extracted (Cho *et al.*, 1991) and blotted to nitrocellulose through a slot-blot apparatus (Sambrook *et al.*, 1989). As a control, RNA from 6 h post-blood meal mosquitoes was blotted as well. These blots were probed with [^{32}P]-labelled cDNA clones for Vg, VCP, or actin (a nonvitellogenic housekeeping gene) as described by Cho *et al.* (1991). Hybridization was quantified by scintillation counting of each sample cut from the blot. A concentration of 10^{-5} M 20E was determined to be optimal (Figure 41.2A).
    b. The minimal concentration of cycloheximide (CHX) necessary to inhibit protein synthesis in cultured fat bodies was determined by preincubating vitellogenic fat bodies for 1 h in complete media containing 0, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, and 10^{-3} M CHX, then pulse-labelling synthesized proteins for 1 h with [^{35}S]-methionine in the continued presence of CHX. Immunoprecipitation of Vg (3.2.1) and TCA precipitation of total proteins (3.2.2) followed by filter radioimmunoassays (3.2.1.a), indicated that >98% of protein synthesis was inhibited by 10^{-4} M CHX.
    c. To demonstrate that the effect of CHX on protein synthesis was not simply caused by cell death, fat bodies were incubated 1 h in complete culture media containing 10^{-4} M CHX, followed by 1 h in media without CHX, and finally 1 h in media containing [^{35}S]-methionine and no CHX. Vg and protein synthesis were assayed as above, and the results showed a recovery in protein synthesis after removal of CHX. Similar experiments were performed to demonstrate that 20E could still stimulate Vg and VCP mRNA transcription after incubation with, and removal of, CHX.
    d. Only after carefully laying this methodological groundwork (a–c) was it possible to perform the definitive experiment (Figure 41.2B): Fat bodies cul-

tured in the presence of both 10^{-4} M CHX and 10^{-5} M 20E did not transcribe Vg or VCP mRNA. CHX was not inhibiting mRNA transcription directly because levels of actin mRNA were unaffected by the presence of CHX. Therefore, the action of 20E on yolk protein gene induction in the fat body must be through at least one intermediary protein, the synthesis of which can be blocked by CHX.

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CHAPTER FORTY TWO

Culture and analysis of insect ovaries

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42.1 INTRODUCTION

In insects, a complex of coordinated processes within the ovary contribute to egg maturation. Among the most important are internalization of large quantities of extra-ovarially produced yolk protein precursors (YPP), massive intra-ovarian synthesis of proteins such as components of chorion, and secretion of ecdysteroid hormones (ecdysteroidogenesis) (Raikhel and Dhadialla, 1992; Dhadialla and Raikhel, 1994; Sappington and Raikhel, 1996). The timing and kinetics of these processes depend on the stage of egg maturation, nutritional status of the female, and environmental conditions. Egg development is mediated through cascades of nervous, endocrine, and intracellular signals. The culturing of insect ovaries is crucial to elucidating the complex web of events comprising egg maturation, as this technique permits the experimental determination and manipulation of individual parameters often difficult or impossible to investigate *in vivo*.

Ovarian culture systems have most commonly been used to study the uptake of YPP. Typically, radiolabelled YPP (Chapter 41) is introduced to the culture medium containing dissected ovaries. Increasing the YPP concentration in the medium demonstrates whether uptake is saturable, a characteristic of receptor-mediated endocytosis. In addition to determining the effect of the oocyte's developmental stage on YPP uptake, this system also allows testing of external variables such as pH, temperature, and divalent cation concentration. Additional insight into the mechanism of YPP uptake is afforded by introducing inhibitors of ion permeability or metabolism into the culture medium. Furthermore, cultured ovaries can be exposed to physiological doses of hormones or second-messengers to elucidate the possible roles of these regulatory molecules in YPP uptake and egg maturation (reviewed in Raikhel and Dhadialla, 1992).

Using this approach, Koller *et al.* (1989) determined that in ovaries of the mosquito, *Aedes aegypti*, maximum uptake of [^{35}S]-Vg occurs 24–30 h post-blood meal (PBM) (Figure 42.1). Moreover, Vg uptake was shown to be temperature-dependent, saturable, selective, and tissue-specific, thus firmly establishing that the accumulation of Vg in mosquito ovaries occurs *via* receptor-mediated endocytosis. Combining decapitation, abdomen-isolation, and blood-enema experiments with ovarian culture, Koller and Raikhel (1991) found that Vg endocytosis and intra-ovarian protein synthesis in the mosquito ovary are activated in two phases: the early phase (com-

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mencing 2–3 h PBM) is controlled by blood-feeding itself, but the initiation and maintenance of the second phase require the presence of a head factor(s) through 8 h PBM for protein synthesis and 16–20 h PBM for Vg endocytosis. In addition, the culturing of previtellogenic ovaries with 20-hydroxyecdysone (20E) revealed that this hormone is not involved in activation of Vg uptake and ovarian protein synthesis (C.N. Koller, T.W. Sappington, P. Helbling, and A.S. Raikhel, unpublished data).

Ovarian culture also has been used to study protein synthesis during egg development. Dhadialla *et al.* (1992) used ligand-blotting to show that the mosquito Vg receptor (VgR) is a 205 kDa protein. To determine VgR substructure, Sappington *et*

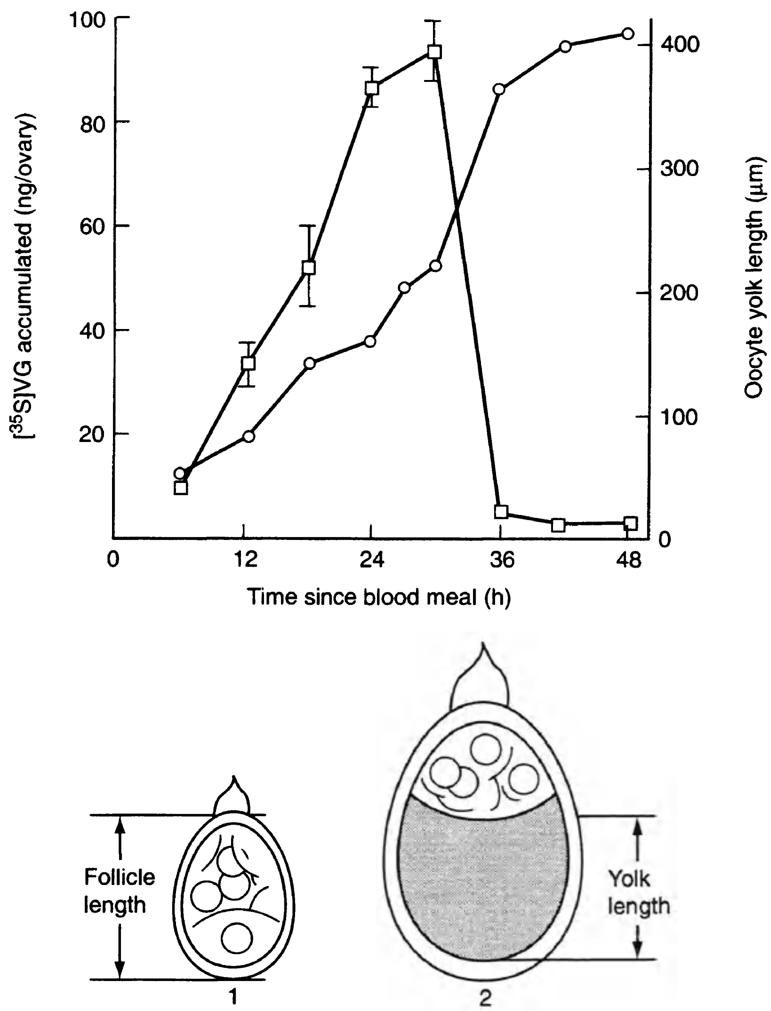


Figure 42.1 (A) Effect of oocyte developmental stage on rate of Vg accumulation by ovaries of *Aedes aegypti* (squares). Size of oocytes was used to determine which ovaries were selected at each time indicated, based on the relationship between hours post-blood meal and yolk length (circles). One ovary each was incubated in wells with 10 μL of uptake Ringer's (Section 42.2.1) and 0.18 μg [^{35}S]-Vg/μL (6.77 × 10^5 cpm/μg). Incubation proceeded for 2 h after a 1 h preincubation period. Points are the mean ± SE of six replicates (from Koller *et al.* (1989) with permission). (B) How to measure follicle length (1) and yolk length (2) in *Ae. aegypti* follicles. Note that all cells in the previtellogenic follicle, i.e. the oocyte, nurse cells, and follicular epithelium, are transparent (1), whereas the vitellogenic oocyte is opaque (2).

al. (1995) cultured 5–8 h PBM ovaries in the presence of a [^{14}C]-labelled amino acid mixture. VgR was partially purified from the ovary extracts and immunoprecipitated using anti-VgR antibodies; the precipitate was separated in a reducing gel, which was then subjected to fluorography to visualize [^{14}C]-VgR. The results demonstrated that the 205-kDa VgR does not consist of subunits (Figure 42.2).

Ovarian culture has been crucial in establishing insect ovaries as the source of ecdysteroids. Application of this technique led to the discovery that vitellogenic *Ae. aegypti* ovaries secrete ecdysone (Hagedorn *et al.*, 1975). Subsequently, Hagedorn *et al.* (1979) used ovarian culture to determine that a peptide factor from the brain, presumably the egg development neurosecretory hormone (EDNH) (Lea, 1967, 1972), stimulates ecdysteroid secretion by the ovary. Matsumoto *et al.* (1989) purified five species of gonadotropic peptides from extracts of *Ae. aegypti* heads and found that at least one of these peptides stimulated ecdysteroid secretion by the ovaries *in vitro*. These peptides

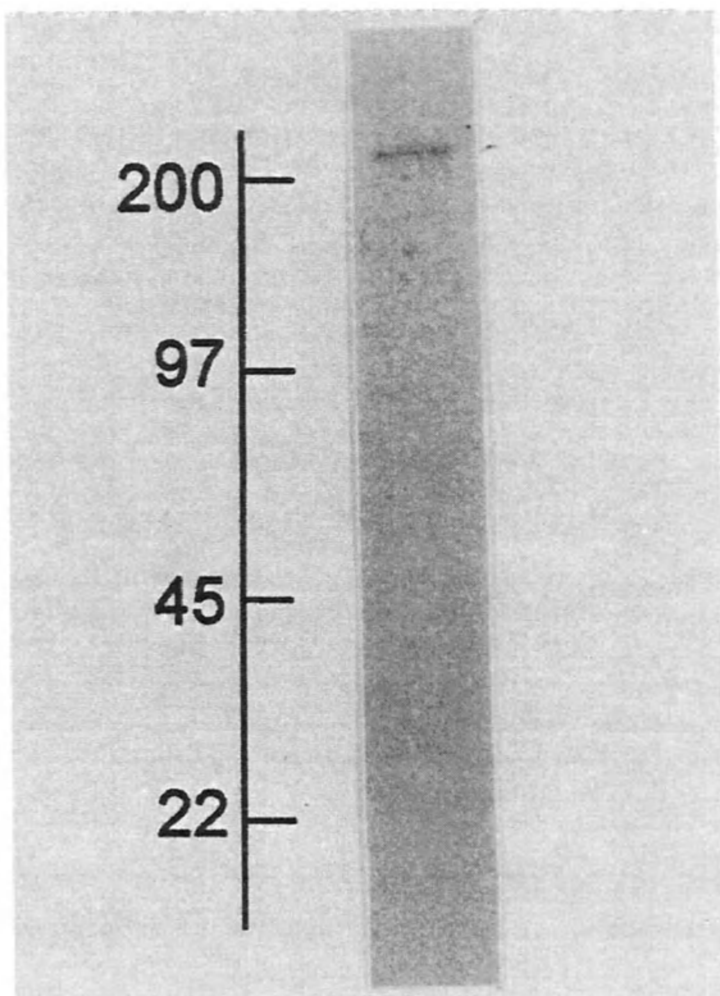


Figure 42.2 Determination of *Aedes aegypti* vitellogenin receptor (VgR) subunit structure. Autoradiograph of [^{14}C]-VgR reduced after immunoprecipitation. [^{14}C]-VgR from 36 processed ovary pairs was incubated with 7.5 μL anti-VgR antibodies in a total volume of 108 μL for 2 h and the protein-antibody complexes precipitated with Protein A-Sepharose. [^{14}C]-VgR was released from the antibodies and reduced by boiling in SDS sample buffer containing 10 mM DTT. Proteins that precipitated were separated by SDS-PAGE in a 1.5 mm-thick, 5–15% gradient gel. After fluorography, Kodak XAR-5 film was exposed to the dried gel for 9 days at –70°C. (From Sappington *et al.* 1995, with permission.)

were termed ovarian ecdysteroidogenic hormones (OEH). Usually in such experiments, ovaries are incubated in the presence of a purified bioactive peptide, secondary messengers, or head extract, and the amount of ecdysteroid secreted into the medium is determined by radioimmunoassay (Shapiro, 1983; Kelly *et al.*, 1986).

In addition, ovarian culture provides a valuable system for investigating gene regulation and expression. Lin *et al.* (1993) incubated ovaries from the mosquito, *Ae. aegypti*, in medium containing 20-hydroxyecdysone (20E) and cycloheximide to study the expression of two vitelline membrane genes. As more ovary-specific genes become available, ovarian culture will increase in significance for the study of gene regulation.

Finally, the insect ovary is a promising target for genetic transformation of insect disease vectors. Presnail and Hoy (1992) reported maternal transformation of the mite, *Metaseiulus occidentalis*, by microinjecting DNA into gravid females. Strategies exploiting receptor-mediated endocytotic pathways for targeted delivery of DNA are receiving increasing attention: the DNA of interest is conjugated to a host ligand specifically recognized and endocytosed by a receptor in the target tissue (Michael and Curiel, 1994). Likewise, DNA conjugated to an insect YPP would be targeted to and endocytosed by a germ cell (the oocyte) and routed to a storage compartment for later release, making this a tempting system to exploit. Clearly, a thorough understanding of receptor-mediated endocytosis of YPP is critical; thus, ovarian culture is an indispensable tool for probing the mechanisms involved.

The following procedures have been used successfully in our laboratories to culture *Ae. aegypti* ovaries and to monitor (i) *in vitro* uptake of Vg, (ii) protein synthesis in general, and VgR synthesis in particular, and (iii) ecdysteroid secretion by the cultured ovaries.

42.2 MATERIALS

42.2.1 Vitellogenin uptake by cultured ovaries

1. Uptake Ringer's: 150 mM NaCl, 4 mM KCl, 0.1 mM $NaHCO_3$, 0.6 mM $MgCl_2$, 25 mM TES buffer. Adjust pH to 7.5 at 27° with NaOH. Add $CaCl_2$ to 3.0 mM. Filter (45-μm pore size). (Store at 4°C, make fresh monthly.)
2. Absolute ethanol.
3. 70% ethanol: dilute with ddH_2O.
4. [^{35}S]-Vg: 3–9 × 10^5 cpm/μg specific activity (Section 41.3.1).
5. TS-2: tissue solubilizer (Research Products International).
6. Aqueous scintillation cocktail: (e.g. Safety Solve, Research Products International).
7. Microtitre plate (e.g. Serocluster U Vinyl Plate, Costar).
8. Whatman 3MM filter paper.
9. Two pairs of super-fine point forceps: e.g. Dumont Biology Grade Tweezers, 110 mm, points 0.05 × 0.01 mm width × thickness.

42.2.2 Total protein synthesis in cultured ovaries

1. Incomplete tissue culture medium: ('incomplete' because it lacks methionine which will be added separately as [^{35}S]-methionine) (Section 41.2.1).

2. APS (*Aedes* physiological saline): 150 mM NaCl, 4 mM KCl, 0.1 mM $NaHCO_3$, 0.6 mM $MgCl_2$, 25 mM HEPES buffer. Adjust pH to 7.0 at 27°C with NaOH. Add $CaCl_2$ to 1.7 mM. Filter (0.45 μm pore size). Store at 4°C. Stable 1 month.
3. Trichloroacetic acid (TCA) stock: TCA-saturated H_2O at 4°C.
4. 10% TCA (i.e. 10% of saturated stock).
5. 5% TCA with 1% nonradioactive methionine.
6. [^{35}S]-methionine (10 mCi/mL) (Note 9).
7. TS-2, tissue solubilizer (Research Products International).

42.2.3 Specific protein (vitellogenin receptor) synthesis in cultured *Aedes* ovaries

1. APS: (Section 42.2.2).
2. APS/suramin: 5 mM sodium suramin in APS.
3. [^{14}C]-amino acid mixture (5 mCi/mL) (Note 9).
4. B2 buffer: 0.5 mM NaCl, 50 mM Tris-HCl, pH 8.4.
5. B2/suramin: 5 mM sodium suramin in B2 buffer.
6. TM buffer: 160 mM NaCl, 2 mM $CaCl_2$, 125 mM Tris-maleate, pH 6.0 at 4°C.
7. CHAPS: 40 mM CHAPS detergent in TM buffer.
8. Protease inhibitors: (Section 41.2.1, step 10).
9. 2× RIPA buffer: (Section 41.2.2).
10. Protein-A sepharose (50% suspension): (Section 41.2.2, step11).
11. 20 mM Tris. Adjust pH to 7.5 with HCl.
12. 2× SDS-PAGE sample buffer: (Section 41.2.2, step13).
13. Sigmacote (or equivalent): for siliconizing surfaces.
14. Spin filter tube: (Section 41.2.2, step 15).
15. RIPA/BSA/GLY buffer: 2% bovine serum albumin, 2% glycine, in 1× RIPA buffer.
16. GF/C Whatman filters.
17. Filtration manifold (e.g. Millipore model 1250 or equivalent).
18. EnHance: (Section 41.2.2, step 23). *Do all work with enhance in a hood.*
19. Coomassie brilliant blue stain (Section 41.2.2, step 24).
20. Formaldehyde fixer (Section 41.2.2, step 25).

42.2.4 Ecdysteroid secretion by cultured *Aedes* ovaries

1. EB (Ephrussi-Beadle Ringers): 129 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 40 mM tricine, pH 6.85.
2. Tris buffer: 50 mM Tris-HCL, 1% BSA, 0.02% sodium azide, pH 7.2 at 4°C.
3. Normal rabbit serum.
4. Ecdysteroid antiserum: Dilute the antiserum 1:20 in ddH_2O with 1% BSA and 1% trehalose. Aliquot and store at –80°C. Freeze and thaw each aliquot only once.
5. Diluted ecdysteroid antiserum: Dilute an aliquot(s) of ecdysteroid antiserum with Tris buffer containing 10% normal rabbit serum to a volume sufficient for the RIA being prepared. This is typically a 1:12–21 000 final dilution for the ecdysteroid antiserum produced and characterized by Kingan (1989).

6. Unlabelled ecdysone: Dispense 20 μL aliquots of ecdysone solution at a concentration of 1000 pg/μL of EB or culture medium. Stable at –80°C for several months.
7. [23,24-^{3}H]-ecdysone (New England Nuclear): Add 100 μL of the radiolabelled ecdysone, as received in solvent, to ca. 10 mL of Tris buffer. Allow the solvent to evaporate. Dilute this solution with more Tris buffer to ca. 14 000 cpm/50 μL. Stable for several months at 4°C.
8. Saturated ammonium sulfate: Prepare 100–200 mL for storage at 4°C. A 1 cm layer of crystals should always be present at the bottom of the bottle. Allow overnight saturation when more ammonium sulfate is added. Shake and allow to settle just prior to use.
9. Aqueous scintillation fluid.

42.3 METHOD

42.3.1 Vitellogenin uptake by cultured *Aedes* ovaries

The following method has been adapted from Koller *et al.* (1989) and Koller and Raikhel (1991).

1. Prepare a microtitre plate: Fill wells with 70% ethanol and let stand 10 min. Rinse wells with absolute ethanol and let dry.
2. Make a stock solution of uptake Ringer's (Note 1) containing 15 μg [^{35}S]-Vg/30 μL of solution (each incubation well will receive 30 μL of total solution) (Note 2), unless [^{35}S]-Vg concentration is the variable being tested. This stock can be split to make the appropriate treatment stocks, or the treatment chemicals can be added directly to the wells.
3. Dispense 30 μL of stock [^{35}S]-Vg/uptake Ringer's into each incubation well. Each incubation well will receive three ovary-pairs.
4. Dissect ovaries (Figure 42.3; Note 3): Anesthetize mosquitoes with ether. Place the mosquito on its dorsal side in uptake Ringer's on a slide under a dissecting microscope. Grasp thorax firmly, but without crushing, with one pair of superfine point forceps (tweezers). With another pair of forceps, tightly grasp the penultimate abdominal segment and pull. The ovaries usually pull out with the abdominal tip. Remove the tip, Malpighian tubules, and excessive tracheae. When working with vitellogenic ovaries, measure the length of the opaque yolk with an ocular micrometer and use only ovaries falling within a range of 20 μm (Note 4).
5. Transfer each ovary-pair to an incubation well (Note 5). Each well receives three ovary-pairs (Note 6). Cover the plate with Parafilm and let stand 2 h at 27°C (Notes 7 and 8).
6. Rinses: For each incubation well, dispense 100 μL uptake Ringer's into each of 3 wells for rinsing. Transfer ovaries from the incubation well into the first rinse well. Carry as little fluid on the forceps as possible. Let stand for 5 min. Transfer the ovaries to the second and third rinse wells and let stand for 10 min each time.
7. Using forceps, place each ovary from a given well onto a very small (e.g. 2–3 mm^2) piece of Whatman 3MM filter paper wetted with uptake Ringer's. Drop the filter (which should have six ovaries [or three ovary-pairs] clinging to it) into

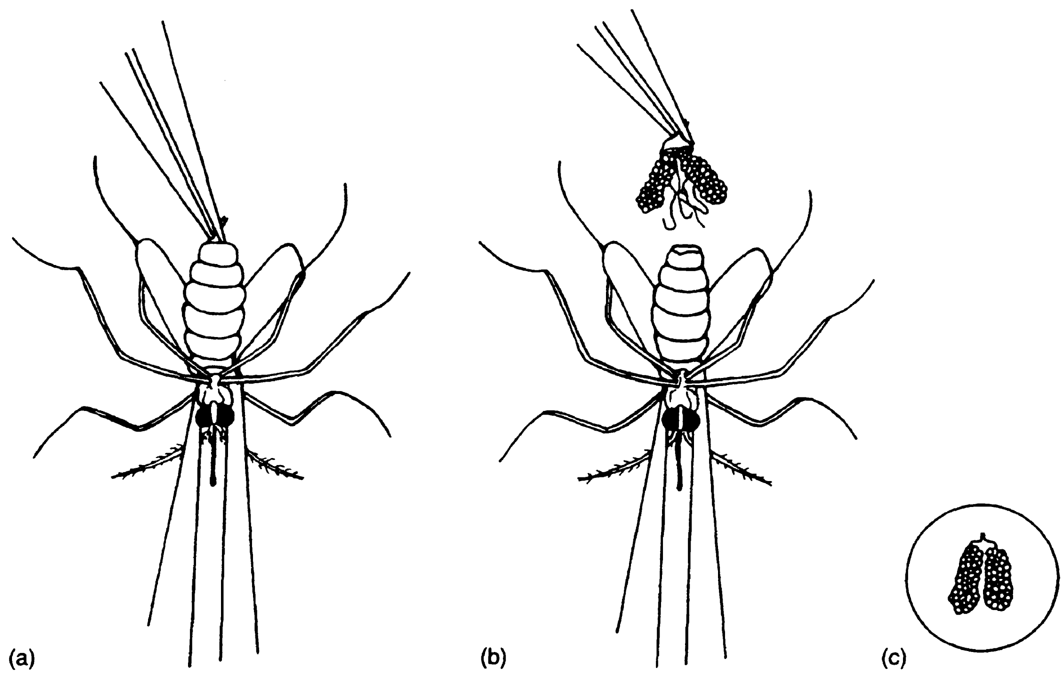


Figure 42.3 Dissection of *Aedes aegypti* ovaries for ovarian culture. (A) One pair of forceps grasps (without crushing) the thorax to hold the mosquito in position while a second pair firmly grasps the penultimate abdominal segment in preparation for pulling out the ovaries. (B) The ovaries are pulled free of the abdomen. Malpighian tubules usually emerge from the abdomen with the ovaries. (C) The ovaries are separated from the abdominal tip (for some applications, it is acceptable to leave the ovaries attached to the abdominal tip) and Malpighian tubules, and are placed in the well of a microtitre plate for incubation. The oviducts connecting the two ovaries are often severed during the dissection.

a scintillation vial containing 75 μL TS-2 tissue solubilizer, and let stand at 37°C for 2 h to overnight.

8. Add 3 mL of aqueous scintillation fluid to each vial, vortex briefly, and place in the dark for 90 min before taking scintillation counts. Be sure to use a blank containing only TS-2 in scintillation fluid, because it tends to produce high background counts.

42.3.2 Total protein synthesis in cultured *Aedes* ovaries

1. Prepare a microtitre plate as described above (Section 42.3.1, step 1).
2. For each ovary-pair, dispense 25 μL of tissue culture medium containing 5×10^5 to 1×10^6 cpm/μL [^{35}S]-methionine (Note 9) into a well.
3. Dissect ovaries in APS as described above (Section 42.3.1, step 4; Note 10). Transfer one ovary-pair to each well: preincubation is not necessary. Cover plate with Parafilm and let stand for 1 h at 27°C.
4. Initial rinses: Transfer each ovary-pair to a well containing 100 μL APS buffer and let stand for 5 min (Note 5). Transfer each ovary-pair twice more to wells containing 100 μL APS buffer and let stand for 10 min each time.
5. Transfer each ovary-pair to a scintillation vial containing 200 μL ice cold 10% TCA and let stand at 4°C overnight. The purpose of this step is to precipitate

(immobilize) the proteins within the ovaries, while unincorporated [^{35}S]-methionine is allowed to diffuse out of the ovaries and into the medium.

6. The next day, draw off the 10% TCA and rinse twice with ice cold 5% TCA containing 1% nonradioactive methionine. Let stand for 15 min each time.
7. After drawing off the last rinse solution, dispense 75 μL TS-2 tissue solubilizer into the scintillation vials containing the ovaries. Let stand for 2 h at 37°C.
8. Add 3 mL of aqueous scintillation fluid and process for scintillation counting as described above (Section 42.3.1, step 8).

42.3.3 Specific protein (vitellogenin receptor) synthesis in cultured *Aedes* ovaries

The following procedure requires polyclonal antibodies to the protein of interest, in this case *Ae. aegypti* vitellogenin receptor (AaVgR). Protocols for immunoprecipitation, filter radioimmunoassay, and SDS-PAGE/fluorography may be found in Sections 41.3.2a, a(i) and b. The following protocol is modified from Sappington *et al.* (1995).

1. Prepare a microtitre plate as described above (Section 42.3.1, step 1).
2. Prepare a stock solution of APS containing 0.2 μCi/μL of a [^{14}C]-amino acid mixture (Note 9). There will be 15 μL of solution per incubation well and six ovary-pairs per well. A total of 36 ovary-pairs from *Ae. aegypti* has given good results (Sappington *et al.*, 1995).
3. Dispense 15 μL of [^{14}C]/amino acid-APS stock into each well.
4. Dissect ovaries from mosquitoes as described above (Section 42.3.1, step 4). Transfer six ovary-pairs to each incubation well. Cover the plate with Parafilm and let stand for 3 h at RT.
5. Transfer ovaries to a well containing 100 μL APS and let stand for 5 min (Note 5). Transfer ovaries to two additional wells of 100 μL APS and let stand for 10 min each time.
6. Draw off the APS from the last wash and replace with 50 μL of APS/suramin buffer containing all protease inhibitors. Transfer ovaries and fluid from all wells to a 2 mL glass-Teflon tissue homogenizer and use a twisting motion with the pestel to homogenize the ovaries.
7. Transfer homogenate to a microfuge tube (Note 11). Rinse the homogenizer with 200 μL of APS/suramin/protease inhibitors and combine with the homogenate. Centrifuge the homogenate at 500 **g** for 10 min at 4°C.
8. Transfer the supernatant to an ultracentrifuge tube and ultracentrifuge at 100 000 **g** for 1 h at 4°C. Discard the supernatant.
9. Resuspend the pellet in B2/suramin buffer containing all protease inhibitors. Ultracentrifuge at 100 000 **g** for 45 min at 4°C. Discard the supernatant.
10. Resuspend the pellet in B2 buffer without suramin but with all protease inhibitors. Ultracentrifuge at 100 000 **g** for 45 min at 4°C. Discard the supernatant.
11. Resuspend the pellet in 30 μL of 40 mM CHAPS. Ultracentrifuge at 100 000 **g** for 1 h at 4°C.
12. Transfer supernatant to a new ultracentrifuge tube and dilute to 15 mM CHAPS with TM buffer. Ultracentrifuge at 100 000 **g** for 15 min at 4°C. Transfer the supernatant to a microfuge tube. Discard the pellet (it may be invisible).

13. Add an equal volume of 2× RIPA buffer to the solubilized AaVgR (i.e. the supernatant from the previous step).

At this point you may proceed with immunoprecipitation of AaVgR followed by filter radioimmunoassay or SDS-PAGE/fluorography as described for Vg in Sections 41.3.2a, a(i) and b, except use RIPA/BSA/GLY buffer in place of RIPA/BSA/MET buffer. (see also Notes 8 and 9 in Chapter 41).

42.3.4 Ecdysteroid secretion by cultured *Aedes* ovaries

This protocol was used by Matsumoto *et al.* (1989).

(a) Ovary culture

1. Dissect five pairs of ovaries (still connected to the last two abdominal segments for ease of transfer) from 3–5 day-old, sugar-fed female mosquitoes and incubate with the test substance in 60 μL of EB (1% BSA and 1% trehalose, optional) or other suitable medium (Note 12) for 6 h at 30°C in a small glass dish or well of a microtitre plate, and put in a humidified chamber (Note 13).
2. After incubation, draw 50 μL of the medium from each ovary preparation and either freeze (-80°C) or immediately assay for ecdysteroid content (Section 42.3.4b). Typically, the media with bioactive substances contain 70–250 pg of ecdysteroids/50 μL, whereas the media from the negative controls contain 30–50 pg/50 μL.

(b) Ecdysteroid radioimmunoassay (RIA)

The incubation media from ovary preparations are assayed for ecdysteroid content with a radioimmunoassay (RIA), modified from that of Warren and Shields (1984). This protocol was used by Matsumoto *et al.* (1989), O'Reilly *et al.* (1992), and Jenkins *et al.* (1992). Because different molecular species of ecdysteroids are present in the incubation media, the experimental results are expressed as pg ecdysteroid equivalents.

1. Prepare and label 6 × 50 mm glass culture tubes, with all samples in triplicate. Tubes A-D are prepared for every RIA to monitor for anomalies and to assure standardization.
   a. Total count (TOT) tubes.
   b. Nonspecific-binding (NSB) tubes: In each tube, dispense 50 μL of 0.05 M Tris buffer containing 10% normal rabbit serum and 50 μL of EB or other culture medium.
   c. Standard zero (STD 0) tubes: In each tube, dispense 50 μL of EB or medium. Data from these tubes provide a check on the antiserum dilution – i.e. the antiserum should bind ~50% of the radiolabelled ecdysone.
   d. Ecdysone standard tubes: Make serial dilutions of unlabelled ecdysone as standards: 1000, 500, 250, 100, 50, 25, 10, 5, or 1 pg/50 μL of EB or medium. Data from these tubes will be used to generate a standard curve.
   e. Experimental sample tubes: Each tube receives 50 μL of sample to be assayed (from Section 42.3.4a, step 2).
2. Add 50 μL of diluted ecdysteroid antiserum to the STD 0, ecdysone standard, and experimental sample tubes.

3. To all tubes, add 50 μL of [23,24-^{3}H]-ecdysone.
4. Vortex all tubes to homogeneity and incubate 18–72 h at 4°C; longer is better, to ensure reaching antibody/antigen binding equilibrium.
5. To all tubes except the TOTs, add 150 μL of saturated ammonium sulfate. Vortex vigorously to homogeneity and let stand 1 h or longer at 4°C.
6. Centrifuge the samples at high speed in a microcentrifuge for 10 min. Check for a small cream-coloured pellet (if the pellet is not visible, see Note 14). Avoiding the pellet, aspirate all of the supernatant from each tube.
7. Add 50 μL ddH_2O to each tube and vortex vigorously to break up the pellet. Add 400 μL aqueous scintillation fluid to each tube and vortex vigorously. A clear homogenous solution should result – if not, this indicates that the ammonium sulfate solution was not completely aspirated from the tube, and the milky solution will affect counting efficiency. Place each tube in a scintillation vial and take scintillation counts for 1 min.

NOTES

1. It is possible to use saline and tissue culture media other than those described here. For example, Kelly *et al.* (1986) assayed ecdysteroid secretion by ovaries of *Aedes atropalpus* and *Drosophila melanogaster* cultured for 5 h. For *Ae. atropalpus,* they used the saline solution of Hayes (1953) (162 mM NaCl, 1.8 mM $CaCl_2$, 2.7 mM KCl, 1.2 mM $NaHCO_3$), and for *D. melanogaster,* they used Ephrussi-Beadle Ringers (Section 42.2.4). See Chapter 41 for a discussion on the importance of using optimized media.
2. It is generally best to perform uptake experiments with saturating concentrations of [^{35}S]-Vg. However, at peak uptake in *Ae. aegypti* (i.e. 24–30 h PBM), this requires a concentration of 8 μg/μL/ovary or 21 μM (Koller *et al.*, 1989), a concentration possible but difficult to obtain. Other insects have even higher saturating concentrations of Vg, e.g., 80 μM in *Nauphoeta cinerea* (Kindle *et al.*, 1988), 73 μM in *Locusta migratoria* (Rohrkasten and Ferenz, 1985), and 30 μM in *Hyalophora cecropia* (Kulakosky and Telfer, 1987). Depending on the variable being tested, less developed ovaries can be used which require a much lower ligand concentration for saturation. For example, at 4 h PBM, saturation occurs at 0.5 μg/μL/ovary in *Ae. aegypti* (Koller and Raikhel, 1991). Shorter incubation times also can help alleviate the effects of nonsaturating concentrations of ligand. Useful comparisons among variables often can be made with less than saturating concentrations of Vg, but this should be taken into account when interpreting results.
3. The small size of insects such as mosquitoes makes separation of individual follicles for culture impractical. However, this is no great disadvantage because after initiation of vitellogenesis with a blood meal, oocyte development is synchronous, making whole-ovary culture possible (Figure 42.3).
4. Because variation in size of the blood meal and in larval development can introduce variability to rates of oocyte development, yolk length within oocytes is a better indicator of equivalent stages of oocyte development than is time PBM (see Koller *et al.*, 1989 and Figure 42.1A for expected ranges of *Ae. aegypti* yolk lengths at different times PBM).

5. All ovary transfers should be done under a dissecting microscope. Ovaries, especially those early in the vitellogenic cycle, often cling to the forceps. Such 'stickiness' tends to increase with each transfer, and forceps tips should be checked after every attempted transfer for clinging ovaries. It is also important to check the receiving well and 'sink' any floating ovaries by tapping them with the point of the forceps. Damaged ovaries should be discarded. Healthy ovaries will continue to wriggle in the Ringer's solution throughout the experiment.
6. It may be advantageous to split the ovary-pair from each female, so that one ovary goes to a treatment well and the other ovary to a control (or paired-comparison) well. This will help reduce variation caused by minor differences among individuals in ovary size or follicle length. If major (rather than subtle) responses to the treatment are expected, this precaution can be omitted. Likewise, in experiments in which comparisons are not being made, such as ligand saturation experiments, there is no reason to split ovaries.
7. It is important to optimize the *in vitro* uptake system for each species tested. For example, Kindle *et al.* (1988) found that *in vitro* Vg uptake by *Nauphoeta cinerea* follicles varied depending on the buffer used in the medium. Koller *et al.* (1989) found the same to be true in the case of *Ae. aegypti* ovaries. The presence of bovine serum albumin in the media improved Vg uptake by *N. cinerea* follicles (Kindle *et al.*, 1988), but it had no effect on uptake by *Ae. aegypti* ovaries (Koller *et al.*, 1989). Calcium ion concentration affects *in vitro* uptake of Vg in *L. migratoria* (Rohrkasten and Ferenz, 1985) and *N. cinerea* (Konig *et al.*, 1988) follicles.
8. In testing the effects of various drugs, hormones, second-messenger chemicals, etc. on YPP uptake, it is important to include a control testing the effects of the solvent. For example, we have found that both ethanol and DMSO can increase uptake of Vg in previtellogenic mosquito ovaries (P. Helbling, T. W. Sappington, and A. S. Raikhel, unpublished data).
9. Although the protocols for total protein synthesis and specific protein synthesis indicate usage of [^{35}S]-methionine and a [^{14}C]-amino acid mixture, respectively, other isotopes, amino acids, or amino acid mixtures may be substituted in both cases. [^{35}S]-methionine is convenient and can be added to an optimized culture medium. However, the specific activity of a [^{35}S]-labelled protein will depend on the percentage methionine in its primary structure. We chose to use a [^{14}C]-amino acid mixture to label *Ae. aegypti* VgR, because we suspected it had a low methionine content (Sappington *et al.*, 1995). One disadvantage to using such a mixture is that the molarities of the individual amino acid components are not optimized for tissue culture in the species being studied. Amino acids labelled with ^{3}H or ^{14}C also can be used individually or in combination, but the specific activity of proteins labelled with these isotopes will probably be low.
10. The best time PBM for dissecting ovaries for protein synthesis depends on the protein of interest. Koller and Raikhel (1991) found that total protein synthesis in *Ae. aegypti* ovaries peaks at 8 h PBM, but remains fairly high through at least 24 h. Vitellogenin receptor accumulation (and likely synthesis) in the *Ae. aegypti* ovary, however, peaks between 18 and 24 h PBM (Sappington *et al.*, 1995).
11. The purpose of steps 7–10 in Section 42.3.3 is to remove most of the vitellin (Vn) (egg storage form of Vg) from the ovary homogenate without removing the AaVgR. Vn is a 'sticky' molecule and will often immunoprecipitate nonspecifically with the AaVgR, reducing the efficiency of this step. AaVgR binds Vn almost as strongly as Vg, and to keep them unbound in the homogenate

requires the presence of suramin. Vn is insoluble in APS but soluble in B2. AaVgR is insoluble in both, but most of it remains suspended in solution during the slow spin in APS while Vn pellets out. These steps were optimized for the special case of separating AaVgR from Vn, so they will have to be modified considerably to accommodate the specific properties of any other protein of interest. If the protein is soluble, a single spin in APS should be all that is necessary to remove Vn and other insoluble proteins before immunoprecipitating.

12. For culturing *Ae. aegypti* ovaries to monitor ecdysteroid synthesis, APS (Section 42.2.2, step2) or complete culture medium (Section 42.2.2, step 1) should work as well as Ephrussi-Beadle Ringers because the former have been optimized for *Ae. aegypti*, whereas the latter is a *Drosophila* saline.
13. Ovaries should be incubated in three or more dilutions of the test substance to determine the upper and lower limits of ecdysteroidogenic bioactivity.
14. Precipitating the immunoglobulins in the rabbit serum with ammonium sulfate is a critical step, because most problems or inconsistencies between RIAs originate at this point. If no pellet forms during centrifugation, this indicates that the ammonium sulfate solution was not saturated. Adding a few crystals of ammonium sulfate and repeating the immunoglobulin precipitation step may save the sample.

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CHAPTER FORTY THREE

Culture and analysis of insect midgut

Hans-Michael Müller and Andrea Crisanti

43.1 INTRODUCTION

Medically relevant mosquito species, such as the malaria vector *Anopheles gambiae* and the yellow fever vector *Aedes aegypti*, have to feed on vertebrate blood in order to initiate vitellogenesis. Together with the blood, they may ingest pathogenic microorganisms such as plasmodia and filariae, or arboviruses. The gut is the organ where the mosquito first comes into contact with these pathogens, and is therefore the object of intense studies regarding parasite-host interactions.

The culture of mosquito derived gut cells would offer the possibility of studying *in vitro* host–parasite interactions, and investigating the effect of exogenous factors on parasite development. Gut culture has already been employed to study physiological aspects of blood digestion. The expression of trypsin, the serine protease mainly responsible for blood digestion in mosquitoes, was analysed in *Ae. aegypti* by *in vitro* pulse–chase experiments (Graf and Briegel, 1989), the effects of blood meal and blood meal size on trypsin expression and secretion were assessed. This experimental system also allowed the effect of juvenile hormone on trypsin expression in *An. albimanus* to be analysed (Hörler and Briegel, 1995). Transfection of cultured mosquito midguts might offer the possibility of functionally characterizing promoters of midgut-specifically transcribed genes, for example the blood meal-induced trypsins of *An. gambiae* (Müller *et al.*, 1993, 1996). These promoters may prove extremely useful for the development of transgenic mosquitoes expressing anti-parasitic agents in the midgut, thereby interfering with malaria transmission.

In this chapter an *in vitro* culture system developed for midguts of adult female mosquitoes and undifferentiated midguts of pupae of *An. gambiae* is described. In addition, the methods used to transfect cultured mosquito midguts are also described. The transfection of mosquito midguts may provide a tool for the study of tissue-specific gene expression, the transfection of the midgut of pupae may be used, in addition, to study gene expression which is linked to differentiation processes.

43.2 MATERIALS

43.2.1 Cell culture

1. Lactalbumin hydrolysate (Sigma).
2. Yeastolate, 200 g/L (JRH Biosciences).
3. Choline chloride (Sigma).
4. Inositol (GibcoBRL).

Molecular Biology of Insect Disease Vectors: A methods manual. Edited by J.M. Crampton, C.B. Beard and C. Louis. Published in 1997 by Chapman & Hall. ISBN 0 412 73660 8.

5. Bovine albumin, fraction V (Sigma).
6. BME vitamins, 100× (GibcoBRL).
7. Fetal calf serum (JRH Biosciences).
8. L-Glutamine, 200 mM (GibcoBRL).
9. Penicillin–streptomycin, 10000 U-10 mg/mL (GibcoBRL).
10. Gentamycin, 10 mg/mL (Sigma).
11. Nystatin, 10 000 U/mL (GibcoBRL).
12. PBS (1 L): 8g NaCl, 2.9 g $Na_2HPO_4.12H_2O$, 0.2 g KH_2PO_4, 0.2 g KCl, pH 7.2.
13. Elastase (Boehringer): prepare stock solution in PBS (10 mg/mL), store at –20°C.
14. 35 mm diameter tissue culture dishes (Falcon 3001).

The above-listed substances are stored as recommended by the supplier. All chemicals not listed are analytical grade, without special preference of supplier.

15. Cell culture media (Table 43.1).
16. Complete medium:
    The complete culture medium is a 1:1 combination of MM or MK and VP12 medium. MM/VP12 is recommended for *Aedes* cells, MK/VP12 for *Anopheles* cells.
    Combine per 100 mL:

MM or MK	45 mL
VP12	45 mL
Fetal calf serum	10 mL
200 mM Glutamine	0.6 mL
Penicillin–streptomycin	1 mL

Store at 10°C, the medium is stable for several months.

43.2.2 Gut transfection

1. Medium without FCS and antibiotics.
2. Lipofectin reagent, GibcoBRL, store at 4°C.
3. Luciferase assay system, Promega, store at –80°C.

Table 43.1 Cell culture media

	MM	*MK*	*VP12*
$NaH_2PO_4.2H_2O$	200	–	550
KH_2PO_4	–	100	–
$MgCl_2.6H_2O$	100	–	1100
KCl	200	500	550
$CaCl_2.2H_2O$	200	100	400
NaCl	7000	6500	3900
$NaHCO_3$	120	100	500
$MgSO_4.7H_2O$	–	–	1200
D-Glucose	4000	4000	2000
Lactalbumin hydrolysate	6500	6500	5000
Yeastolate	25 ml	25 ml	–
Choline chloride	–	–	250
Inositol	–	–	400
Bovine albumin	–	–	1000
BME vitamins	–	–	20 ml

Where not otherwise indicated, the weight of the substances is given in milligrams. Prepare the media separately and bring them to a final volume of 1 l. Adjust to pH 7.0 with 2% KOH if necessary. Filter sterilize and store at 4°C, the media are stable for several months.

43.3 METHODS

43.3.1 Gut dissection

Dissections are carried out under a stereo microscope, using 40× magnification. Usually, batches of 20 pupae or adult mosquitoes are dissected together.

(a) Dissection of pupae midgut

1. Collect the pupae with an inverted Pasteur pipette and put them into a small volume (20 mL) of the same water in which they were raised; in the case of *An. gambiae* this would be 0.1% marine salt dissolved in distilled water. Add penicillin (100 U/mL), streptomycin (100 μg/mL), and gentamycin (100 μg/mL).
2. Transfer the pupae, together with the water, into a Petri dish with a filter paper inlay. Remove enough water so that the pupae are still able to move but can also be picked up easily with the needle.
3. Divide 100 μL of complete medium into three separate drops on a slide and put it under the microscope. Focus on the tip of the dissection needle (Note 1).
4. Pick up one of the pupae by piercing the thorax with the needle and put it into the first drop of medium.
5. Remove the head, open the thorax, and squeeze out the gut by pressing alternately with both needles on the abdomen, beginning at the posterior part.
6. Move the gut away from the carcass, at the edge of the drop, and cut off the Malpighian tubules, then transfer the gut into the next two drops of medium in order to wash it out. In the third drop, move the gut to the edge, carefully take it up with both needles, and control which tip it adheres to.
7. Transfer the gut to a microfuge tube containing 0.5 mL complete medium by submerging the needle a few mm and at the same time agitating it. Do not touch the wall of the tube with the tip of the needle. Observe how the gut separates from the tip of the needle and sinks down to the bottom of the tube, then close the tube again.
8. Clean the slide with a wet paper towel and continue with step 3. With some practice, the dissection of one gut will take not longer than 2 min.

(b) Dissection of mosquito midgut

1. Collect 20–30 female mosquitoes in a cup which is covered with gauze.
2. Anaesthetize the mosquitoes by putting the cup at –20°C for 30 s.
3. Quickly transfer the immobilized mosquitoes to a Petri dish on humidified filter paper, and cover it. The Petri dish may be kept on ice and each mosquito decapitated just before dissection. Alternatively, after a few minutes on ice, all mosquitoes may be decapitated and then kept at RT.
4. Divide 100 μL of complete medium in three separate drops on a slide and put it under the microscope. Focus on the tip of the dissection needle (Note 1).
5. Pick up a mosquito by piercing the thorax with the needle and put it into the first drop of medium.
6. Leave the needle in the thorax, press the tip of the second needle on the second or third last abdominal segment and slowly pull out the gut.
7. Cut off the tube-like anterior midgut and the Malpighian tubules together with the hindgut, and wash the midgut in the two remaining drops of medium.
8. Continue with step 7, Section 43.3.1a.

(b) Dissection of midgut from blood-fed mosquitoes

To dissect the midguts from blood-fed mosquitoes, proceed as described in Section 43.3.1b, with the following modifications:

1. A few hours after blood-feeding, the gut epithelium is still highly extended and the peritrophic membrane has not yet formed. At this stage the midgut is very fragile and may not withstand the tension when trying to pull it out of the abdomen. To dissect an intact midgut shortly after blood-feeding, try to remove as many abdominal segments as possible and pull out half of the midgut. Then cut off the thorax, and carefully squeeze out the rest of the gut by pressing the needle on the anterior part of the abdomen.
2. It is much less problematical to dissect midguts of mosquitoes that were fed more than 12 h before use. The blood volume is substantially reduced, the blood bolus coagulated, and the peritrophic membrane formed. However, at this time the Malphigian tubules start to fill up with bacteria. In order to minimize bacterial contamination of the cultures, it is necessary to carefully separate the Malpighian tubules from the guts without injuring them. The easiest way to achieve this is to sacrifice a small part of the posterior midgut and to move the midgut away from the carcass as fast as possible.
3. Usually the content of the blood meal is removed before culturing the guts. This procedure is carried out as follows: after transferring the gut to the second drop of medium, open it laterally and wait until the gut epithelium shrinks together on the opposite site of the blood bolus. Carefully remove the blood bolus and remove residual blood attached to the gut by swirling the epithelium in the drop of medium with the needle. Repeat this in the third drop of medium, before transferring the gut to the microfuge tube.

43.3.2 Gut culture

(a) Standard procedure

For mosquito gut culture a variety of media can be used, the most common are MM (Mitsuhashi and Maramorosch), MK (Modified Kitamura) and VP12 (Varma and Pudney) which may be combined to yield optimal results (Pudney *et al.*, 1979; Varma *et al.*, 1979). Grace's medium (Graf and Briegel, 1989) and Reddy's medium (Morris *et al.*, 1995) may also be used for mosquito cell culture. The cultures are kept at 27°C, without the need of a specialized incubator. However, the cultures should be closed securely to avoid the loss of CO_2 from the medium. Pulse-chase experiments during short-term midgut culture can be performed using microfuge tubes or microtitre plates (Graf and Briegel, 1989). When culturing a larger number of guts, for example 20, use 35 mm diameter tissue culture dishes (Falcon 3001). The following procedure is recommended for the long-term culture (up to 3 weeks) of midguts.

1. During dissection, collect guts in a microcentrifuge tube containing 0.5 mL of complete medium.
2. Spin down the guts for a few seconds. From now on work in a sterile hood.
3. Remove the supernatant and add 200 μL of fresh medium (see also Section 43.3.2b).
4. Fill a 35 mm diameter tissue culture dish with 1.6 mL of medium.

5. Carefully transfer the 200 μL medium containing the guts in the culture dish. Use a 1 mL pipette tip widened by using a sterile blade, in order to retain the guts intact.
6. Wash the tube with 200 μL medium to remove gut residues and add it to the culture.
7. Put the sealed tissue culture dishes in a humidified, tightly closed plastic box, and incubate at 27°C.

(b) Treatment of dissected mosquito gut with elastase

A fraction of pupae guts (about 10%) will attach to the plastic surface of the culture dish. We have not observed any attachment of midguts from 1-day-old pupae or adult mosquitoes. The attachment can be increased by up to 30% by digesting the basal lamina of the pupae guts with elastase. Carry out the elastase treatment according to the following procedure (which replaces step 3, Section 43.3.2a):

1. Replace the medium in which the dissected guts were collected with 200 μL of medium, without FCS but containing 2 mg/mL elastase, prepared from a 10 mg/mL elastase stock solution in PBS.
2. Incubate 30 min at RT; gently mix every 5 min.
3. Spin down the guts and replace the supernatant with complete medium. Repeat this wash once.
4. Continue with step 4, Section 43.3.2a.

From attached guts, cells grow out during the following days (Figure 43.1), and a large number of vesicles form within the cells which have spread on the plastic surface. It is not yet clear whether the cells only spread out of the gut, or whether cell division also occurs. However, the observation that the undifferentiated pupae gut can be transformed more efficiently than midguts of adult mosquitoes, for example with an actin-luciferase reporter plasmid (Section 43.3.3a), indicates that at least some initial cell division occurs. Peristaltic movements of the cultured tissue may be observed for up to 3 weeks in those guts dissected from adult mosquitoes and pupae. In the case of the guts from pupae, these movements are restricted mainly to attached guts.

43.3.3 Analysis of cultured midgut

Midgut culture has proven to be a useful system for studying several aspects of mosquito biology including: (i) protein synthesis of digestive enzymes (Graf and Briegel, 1989); (ii) secretory processes (Graf and Briegel, 1989); and (iii) effects of externally added molecules like hormones (Hörler and Briegel, 1995). Midgut culture could potentially be used to test the effect of substances on *Plasmodium falciparum* development under defined conditions *in vitro*. The use of pupae guts as a culture system has additional advantages including: (i) the possibility of following developmentally linked processes, for example the onset of constitutive trypsin synthesis in *Anopheles*, and (ii) the transfection of midguts *in vitro*. This last procedure may offer the possibility of examining the promoters of genes, such as trypsins, which are specifically transcribed in the midgut. In this section, a protocol for transfection of mosquito guts is described.

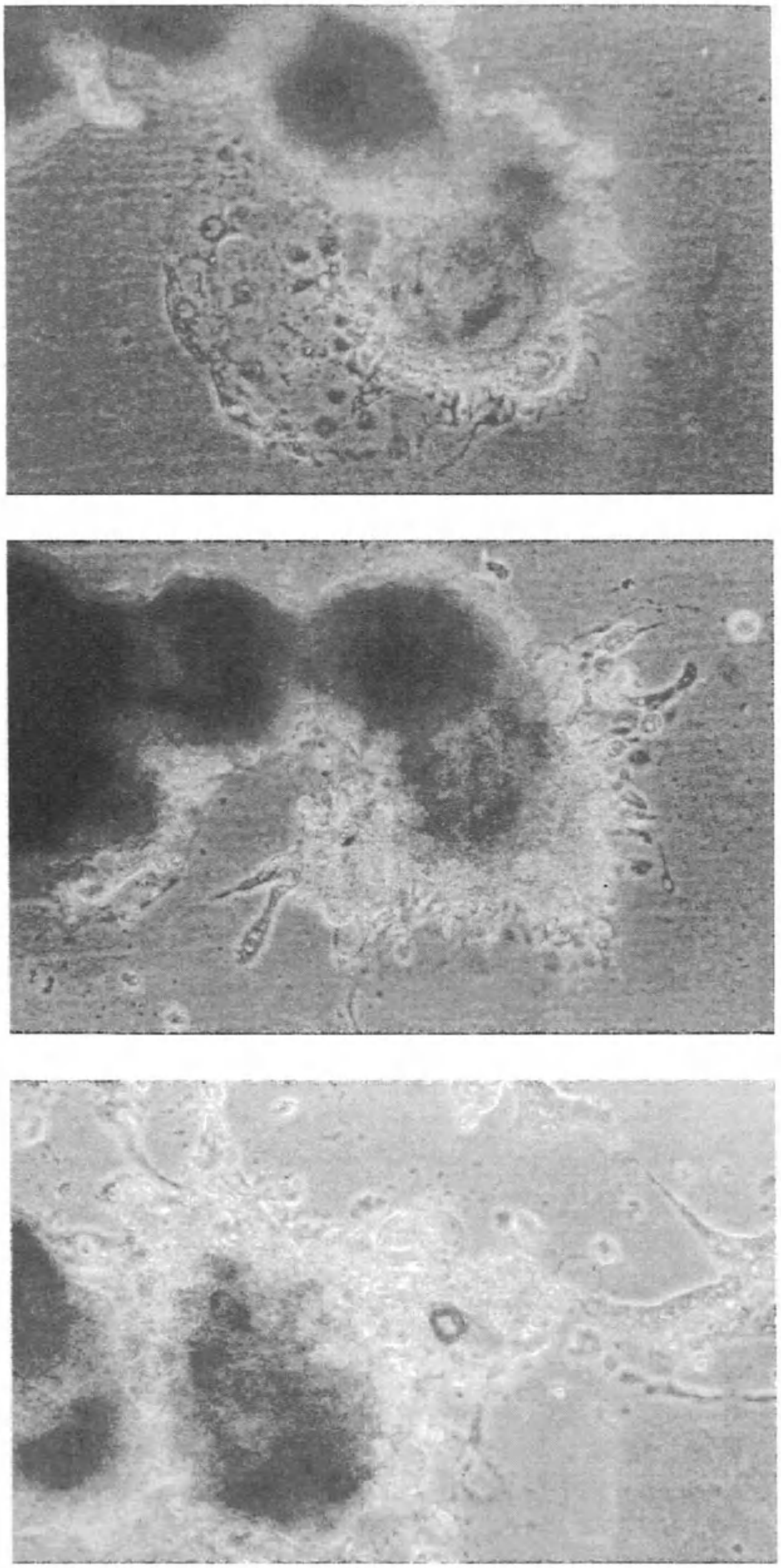


Figure 43.1 Phase contrast micrographs of midguts of teneral pupae of *Anopheles gambiae*, after (A) 2 days, (B) 8 days, and (C) 16 days in culture.

(a) Transfection of midguts
Transfection experiments have been performed on pupae guts in lipofectin-conditioned medium, using a series of reporter constructs. The efficiency of the transfection was monitored by measuring the activity of the reporter gene luciferase. Efficient transfection of *Ae. aegypti* salivary glands was shown to depend on the removal of the basal lamina by elastase (Morris *et al.*, 1995). In the case of midguts from *Anopheles*, no improvement has been observed when applying this technique. A reason for this might be the more resistant basal lamina which surrounds the gut in *An. gambiae.* However, transfection efficiency could be improved several-fold by cutting the guts into pieces. Lipofectin was used as the transfection reagent. The plasmid constructs used for transfection contained the luciferase gene under the control of the *D. melanogaster* actin or heat shock promoter, or the upstream regions of the *An. gambiae* trypsin genes. The negative control used was the luciferase reporter construct without a promoter. The Luciferase Assay System of Promega was used to measure luciferase activity in a Berthold Lumat LB9501.

1. Put 10–20 dissected, washed guts in a microcentrifuge tube containing 100 μL of medium without FCS and pipette up and down 20 times. This ruptures the guts, yielding pieces of tissue and cell clumps.
2. Transfer the gut pieces to a cell culture dish filled with 1.8 mL of medium without FCS (Note 2) and wash out the tube with a further 100 μL of the same medium.
3. Prepare two microcentrifuge tubes, each containing 40 μL medium without FCS.
4. Add 10 μL of plasmid DNA (5 μg) to the first tube and 10 μL of Lipofectin to the second tube.
5. Transfer the Lipofectin-mix to the tube containing the DNA, and mix gently.
6. Incubate 15 min at RT, then pipette the DNA/Lipofectin-mix to the culture dish and mix by gentle agitation.
7. Add gentamycin to a final concentration of 100 μg/mL, then incubate for 18 h at 27°C.
8. Replace 80% of the supernatant with fresh, complete medium containing antibiotics (Notes 3 and 4).
9. Allow the cells to recover for 8 h at 27°C, then collect the guts by centrifugation, lyse the cells and measure the luciferase activity according to the manufacturer's instructions.
10. When using the heat shock promoter, allow the guts to recover for 3 h at 27°C, then incubate them for 90 min at 41°C and allow them to recover for 3 h at 27°C.

Performing this transfection procedure with 15 guts of teneral pupae using the *D. melanogaster* actin or heat shock promoter constructs, we have measured a luciferase activity of 50- to 200-fold over background (unpublished data). However, values of only three-fold above background were observed when transfecting differentiated midguts of female mosquitoes. In order to assess whether this system might be suitable to measure tissue-specific promoter activity, we have used the upstream regions of the *An. gambiae* trypsin genes *Antryp1* and *Antryp4*, fused to the luciferase gene. *Antryp1* is a mainly blood meal-induced trypsin, which is already expressed in the gut of pupae, whereas *Antryp4* is constitutively expressed soon after emergence (Müller *et al.*, 1995). When transfecting guts of pupae, both constructs yielded luciferase activity corresponding to about 25% of the value obtained with the heat shock promoter construct. Luciferase activity was also observed when transfecting guts of blood-fed mosquitoes with the *Antryp1* and *Antryp4* constructs. However, the values obtained were less significant than in the case of pupae guts.

Clearly, the transfection of insect gut is not yet a well established technique, and we are currently working on the optimization of the protocols presented here. The use of other proteases, in addition to elastase for digestion of the basal lamina, variations in the amounts and ratios of DNA and Lipofectin, whose optimal concentrations could well differ from those used for established insect cell lines, might lead to substantial improvements in the efficiency of transfection.

NOTES

1. Dissections may be carried out in buffered saline, such as PBS, or in cell culture medium. The latter has advantages especially when dissecting pupae, as it generally facilitates the dissection and renders the gut less sticky, therefore facilitating the transfer of the gut with the dissection needle into the tube containing the medium.
2. During transfection, the presence of FCS and antibiotics is not usually recommended. However, we have not observed significant effects when transfecting an *Ae. aegypti* cell line (Mos20) or midguts in the presence of antibiotics. However, the presence of FCS reduces the transfection efficiency several-fold.
3. When used for the growth of insect cell lines, the medium usually contains penicillin and streptomycin. In order to suppress bacterial contamination in midgut cultures, the additional use of gentamycin (100 μg/mL) is recommended. In short-term cultures, for example transfection experiments, fungal infections commonly do not pose a problem. If they occur, they can be controlled effectively by adding nystatin to the cultures (100 U/mL).
4. When using mosquito guts in long-term culture, there will probably be problems with bacterial contamination, especially with guts from blood-fed mosquitoes. We have tried to decrease the bacterial input of the mosquitoes by feeding them on sugar solution containing penicillin (100 U/mL), streptomycin (100 μg/mL), and gentamycin (100 μg/mL) from emergence onwards, and had some success. However, careful dissection without injury to the Malpighian tubules and thorough washing of the guts in medium, seems to be the best way to keep down bacterial contamination. In contrast, guts from pupae never posed sterility problems. We have actually never observed contamination of pupae gut cultures with bacteria, and rarely with fungi. It may well be that an elevated level of antibacterial peptides in pupae are the reason for this.

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Part Eight
Insect Symbionts

CHAPTER FORTY FOUR

Isolation and culture of tsetse secondary endosymbionts

Susan C. Welburn and Colin Dale

44.1 INTRODUCTION

Insect symbionts have attracted attention recently for their potential use as a vehicle for the expression of anti-parasitic gene products in arthropod disease vectors (Beard *et al.*, 1993; Aldhous, 1994). Much research has focused on a bacterial endosymbiont found in the haemolymph and various tissues of the tsetse fly (*Glossina* spp.). This symbiont, referred to as 'rickettsia-like-organisms' (RLO) or 'secondary symbionts' are present in some tsetse with two other bacterial endosymbionts: the mycetome bacteroids and *Wolbachia* spp. The giant bacteroids of tsetse are found exclusively within the midgut mycetome and are believed to provide essential vitamins required to supplement the fly's haematophagous diet (Nogge, 1978). *Wolbachia* spp., found only in ovarian tissues, have been implicated as agents of 'cytoplasmic incompatibility' in many insects including tsetse and it has been suggested that this phenomenon could be utilized to drive engineered symbionts through wild fly populations (Aldhous, 1994).

The 'rickettsia-like organisms' of *Glossina* spp. have been isolated and cultivated in a mosquito cell line (Welburn *et al.*, 1987) and remain the only 'secondary' endosymbiont to be cultivated *in vitro*. These organisms are associated with maternally inherited susceptibility to trypanosome infection in tsetse (Maudlin *et al.*, 1986) which is thought to depend on the production by symbionts of significant quantities of chitinase (Welburn *et al.*, 1993). In an ultrastructural study of the mycetome of several different tsetse species Reinhardt *et al.* (1972) observed RLO in the cytoplasm of surrounding muscle cells. Pinnock and Hess (1974) found small rod shaped RLO randomly located within the cytoplasm of midgut cells of four species of tsetse and RLO were observed in both wild caught and laboratory reared flies. RLO were also found in the mycetome and midgut epithelium, in cells closely associated with the fat body and also in oocytes. Pinnock and Hess (1974) failed to detect RLO in spermathecae, salivary glands, undeveloped ovary, flight muscle, malpighian tubules and thoracic ganglion tissues. In the older literature RLO were frequently confused with *Wolbachia* spp., endosymbionts present in the ovaries and so reports of the presence of these microorganisms by Huebner and Davey (1974) in oocytes and nurse cells of the adult tsetse ovary remain unsubstantiated.

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Using 16S rRNA sequence analysis, the phylogenetic position of both RLO and *Wolbachia* spp. from different species of *Glossina* has been clarified; RLO are now considered to belong to the gamma subdivision of the Proteobacteria (Enterobacteriacae) (Beard *et al.*, 1993) showing strong sequence homology with many mammalian enteric bacteria – while *Wolbachia* spp. organisms, present in gonadal tissues were found to group within the alpha subdivision of the Proteobacteria, along with the Rickettsiacae (O'Neill *et al.*, 1993). The midgut mycetome bacteroids (primary endosymbionts of tsetse) have been grouped within the gamma subdivision of Proteobacteria but were distinct from RLO which shared high sequence homology with other 'secondary' endosymbionts from the flour beetle *Sitophilus zeamais*, and the aphid *Acyrthosiphon pisum* (Aksoy *et al.*, 1995).

RLO are common in blood-sucking insects, but detailed study and classification of these microorganisms has been impeded through a lack of culture systems (Dasch *et al.*, 1984). The inheritance of these endosymbionts in tsetse has only recently been elucidated. Roubard (1919) and Wigglesworth (1929) originally suggested that the bacterial symbionts of the midgut mycetome were transmitted through the milk gland, but the existence of bacteria in the milk gland and ducts was not verified until much later. Ma and Denlinger (1974) observed bacteroids in the milk glands of several tsetse species, as did Bonnanfant-Jais (1974) in *Glossina austeni* Newstead. Pell and Southern (1976) showed that RLO were inherited transovarially, but also suggested that they could be passed on to larvae through larval milk. Susceptibility to trypanosome infection in tsetse has similarly been shown to be a maternally inherited character (Maudlin *et al.* 1986) and a study of wild and laboratory flies has associated RLO numbers with the degree of fly susceptibility (Maudlin and Ellis, 1985; Maudlin *et al.*, 1990). The culture of these microorganisms has provided a means of investigating and potentially manipulating the mechanism involving parasite transmission by tsetse.

The methods detailed here will allow the investigator to isolate and maintain RLO from different *Glossina* species and possibly other arthropod disease vectors. We present methods for: (i) the isolation of symbionts from flies using the coverslip culture technique (Weiss and Dressler, 1958); (ii) the maintenance of symbionts in an *Aedes albopictus* cell line and transfer to axenic growth; (iii) the identification and verification of symbionts *in vivo* and *in vitro* by staining, polymerase chain reaction (PCR) and extrachromosomal DNA analysis.

44.2 MATERIALS

44.2.1 Reagents for isolation and culture of endosymbionts

1. Mitsuhashi and Maramorosch Basal Medium (MM): can be obtained commercially (Flow Laboratories, Rickmanworth, UK) or made up with the ingredients below. The following reagents are dissolved in a total volume of 1 L:

    8.125 g Lactalbumin hydrolysate
    6.250 g Yeastolate
    0.250 g $CaCl_2.2H_2O$
    0.250 g KCl
    0.125 g $MgCl_2.6H_2O$
    8.750 g NaCl

0.150 g $NaHCO_3$
0.2826 g $NaH_2PO_4.2H_2O$
5.0 g D-Glucose

The above reagents are dissolved in a total volume of 1 L ddH_2O, filter sterilized, and supplemented with 20% fetal calf serum (FCS) (Gibco, Uxbridge, UK).

2. Alcide: commercially available (Life Science Laboratories, Luton, UK).
3. *Aedes albopictus* cell line: maintained in MM medium with 20% fetal bovine serum.
4. Phosphate buffered saline (PBS) pH 6.8.

44.2.2 Reagents for staining endosymbionts

1. Concentrated carbol fuchsin:
   20 g Basic fuchsin
   200 mL Absolute ethanol
   100 g Phenol
2. Dissolve the basic Fuchsin in the phenol in a beaker in a 56°C water bath. Add the absolute ethanol, complete the volume to 2 L with ddH_2O, and filter through a Whatman No. 1 filter.
3. 1% (v/v) Aqueous glacial acetic acid.
4. 2% Malachite green: 50 g malachite green in 2.5 L ddH_2O.
5. 1% (w/v) Aqueous crystal violet.
6. Lugol's iodine: 1 g iodine and 2 g KI in 100 mL ddH_2O.
7. 1:1 Acetone:ethanol solution.

44.2.3 Reagents for isolation of plasmid DNA from endosymbionts

1. Plasmid purification kit: commercially available. The Wizard miniprep plasmid DNA kit works very well (Promega); however, similar kits from many other companies may work just as well.

44.3 METHODS

44.3.1 Isolation and cultivation of symbionts

RLO are distributed in many tsetse tissues, but for culture purposes are most easily obtained from the haemolymph of teneral (unfed) tsetse flies. RLO have been cultured from haemolymph of the following tsetse species: *Glossina morsitans morsitans, G. m. centralis, G. m. submorsitans, G. palpalis palpalis, G. tachinoides, G. austeni, G. pallidipes, G. p. gambiensis* and *G. fuscipes fuscipes*. Before isolation and cultivation of symbionts can take place, it is necessary to set up an *Ae. albopictus* cell line which can then be infected with RLO obtained from the haemolymph of surface sterilized flies. Cultures should be passaged weekly and checked for contamination using blood agar plates and Gimenez staining. Axenic serum-free culture is useful particularly when extracellular enzymes need to be purified.

(a) Primary cultivation of RLO

1. Surface sterilize at least 20 newly emerged tsetse using three changes of Alcide L.D., 10 min for each change.
2. Dry the flies on sterile filter paper in a cabinet.
3. Isolate fly haemolymph by puncturing each fly in the fons region of the head using a sterile needle and collecting the haemolymph in sterile 2 μL micropipettes (Camlab, Cambridge, UK). Dispense haemolymph into a sterile microfuge tube but do not chill.
4. When collection is complete the haemolymph should be pipetted into a minimum volume (0.5 mL) of MM culture medium supplemented with 20% FCS and divided between three flat-bottomed centrifuge tubes containing circular coverslips (1 cm diameter) previously seeded with *Ae. albopictus* cells.
5. Centrifuge each tube at 500 g for 10 min at 25°C and place in a cooled incubator set at 25°C.
6. At 7-day intervals the tubes should be agitated by vortexing and the cells subpassaged by transferring 100 μL of the suspension onto confluent *Ae. albopictus* cells. Cultures should be monitored by Gimenez staining (described below) and checked for contamination using blood agar plates. To do this, streak a loopful of cell suspension onto the surface of a blood agar plate and incubate at 37°C overnight (most contaminants will grow on this rich medium). Cultures of RLO may be expanded by subculture into larger tissue culture flasks seeded with *Ae. albopictus* cells. For culture volumes over 50 mL we recommend that axenic cultivation be used.

(b) Separation of RLO and host cells

For preparation of RLO it is best to use cultures that are at least 96 h old. This ensures that most of the host cells have lysed leaving the bacteria free in the supernatant.

1. Vortex the cell suspension to free any clusters of cell debris.
2. Pellet any remaining intact host cells by centrifugation at 500 g for 10 min at 25°C.
3. Recover the supernatant and pellet the RLO by centrifugation at 20 000 g for 25 min at 25°C.
4. For DNA/protein preparation the cells may be re-suspended in PBS pH 6.8 and re-centrifuged.

(c) Axenic cultivation of RLO

1. Inoculate MM medium (with or without 20% FCS, Note 1) with purified RLO from an *Ae. albopictus* culture. We recommend that at least a 1/20 volume inoculum from a 96 h culture is used.
2. Measure the initial turbidity of the culture using a spectrophotometer at A_{600}.
3. Incubate at 25°C and after three days check the turbidity at A_{600} (expect a 2-fold increase for a healthy culture).
4. Subculture a 1/20 vol every week and check for contaminants as outlined above.

44.3.2 Staining techniques

Gimenez (1964) developed a technique for determination of rickettsiae from yolk sac material by incorporating carbol Fuchsin in the buffer and using malachite green as

a direct counterstain. Using this modified technique, rickettsiae stain bright red while the yolk sac cells appear as a contrasting blue-green. This stain also works well with RLO, especially with material from flies (e.g. haemolymph) or *Ae. albopictus* cell cultures. The Gram stain is quick and easy and is best used to stain RLO from axenic cultures. The pleomorphic nature of these small Gram-negative organisms makes identification relatively easy (Figure 44.1).

(a) Gimenez stain

1. Spot 10 μL samples of haemolymph or culture fluid onto glass slides, dry in air for 1 h and gently heat fix the slides in a Bunsen flame.
2. Stain for 12 min with carbol fuchsin (diluted 1:10 with distilled tap water).
3. Rinse in tap water and decolorize with 1% aqueous glacial acetic acid for 5–10 s.
4. After rinsing again in tap water, counterstain with 2% malachite green for 1 min.
5. Rinse well with tap water and dry the slides in air before microscopic examination. RLO stain pink, other cells stain green/blue (Figure 44.1 for reference).

(b) Gram stain

1. Prepare slides as for Gimenez staining.
2. Stain for 1 min with 1% aqueous crystal violet.
3. Wash with Lugols Iodine for 1 min.
4. Decolorize for 5 s with 1:1 acetone:ethanol.
5. Rinse slides in tap water and counterstain for 2 min with carbol fuchsin (diluted 1:10 with distilled water).

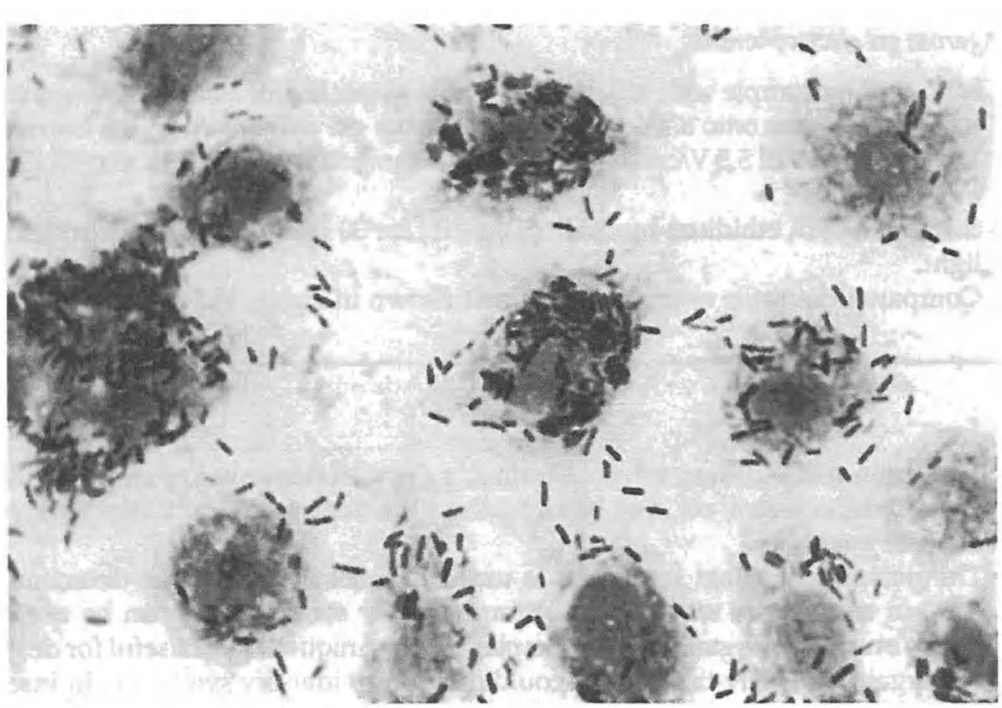


Figure 44.1 *Ae. albopictus* cells 72 h post-infection with RLO and stained by the method of Gimenez (1964).

6. Rinse well with tap water and dry the slides in air before microscopic examination. Gram-negative RLO stain pink, Gram-positive bacteria stain blue.

44.3.3 Extrachromosomal DNA analysis

RLO carry an array of large extrachromosomal DNA elements which can be purified and examined by agarose gel electrophoresis. Along with staining, analysis of extrachromosomal DNA provides the most convenient means for checking the integrity of cultures. We have found that the Promega Wizard minipreps system gives the most consistent results. The technique is easy to perform, uses very little starting material and can be carried out in under an hour. Alternatively, it may be possible to use one of the general methods for plasmid purification as described in Sambrook *et al.* (1989). Pulsed-field gel electrophoresis is the best method for analysis of extrachromosomal DNA, although low percentage agarose gels provide enough resolution for diagnostic purposes.

(a) Preparation of extrachromosomal DNA

1. Isolate RLO from a 15 mL 96 h *Ae. albopictus* culture, or 10 mL late log-phase axenic culture.
2. Resuspend the bacteria and prepare extrachromosomal DNA as outlined in the Wizard minipreps instruction manual.
3. Elute the DNA from the column with 50 μL of water heated to 70°C for optimum yield. Store the sample in the dark at 4°C and electrophorese within the next 2 h. Minimize exposure to strong UV light as this causes a shift in migration of the circular DNA molecules.

(b) Agarose gel electrophoresis

1. Mix 20 μL of sample with 10 μL of 4× DNA sample buffer and load along with a λ *Hind*III marker onto a 1% pulsed field agarose gel without ethidium bromide.
2. Electrophorese at 5.5 V/cm with switch time ramped between 0.2 s and 13 s over a 15 h period.
3. Stain the gel in ethidium bromide (5 μg/mL) for 30 min and visualize under UV light.
4. Compare the profile obtained with those shown in Figure 44.2.

NOTES

1. The absence of FCS is an advantage during extracellular protein purification and agglutination assays etc., but often leads to the demise of the culture after two or three passages.
2. The polymerase chain reaction is a useful diagnostic test for the detection of RLO *in vivo* and *in vitro*. The reaction is highly sensitive and can be used to detect only a few organisms in a sample. This technique is most useful for detecting organisms in fly tissues and could be used to identify symbionts in insects other than tsetse. The PCR reaction is not suitable for monitoring the integrity of

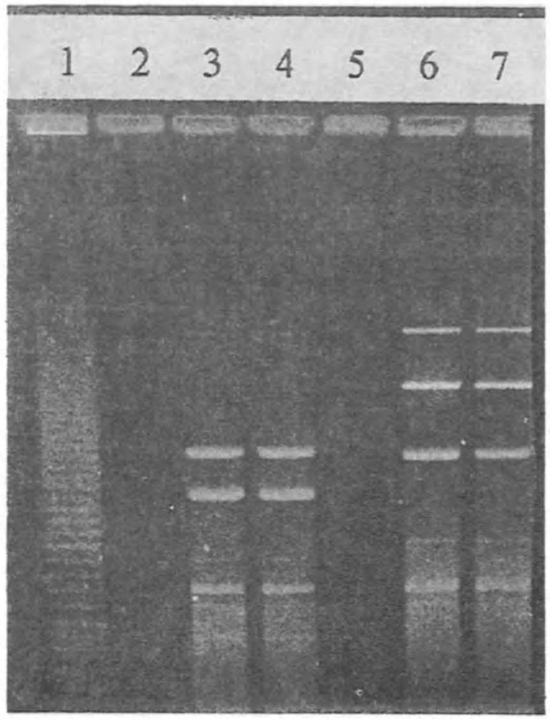


Figure 44.2 Pulsed-field gel electrophoresis of RLO extrachromosomal DNA using a Bio-Rad CHEF DR-II. Lane 1: ø×174 5.4 kB concatamer ladder. Lanes 3 and 4: Extrachromosomal DNA from *G. p. palpalis* RLO. Lanes 6 and 7: Extrachromosomal DNA from *G. m. morsitans* RLO.

cultures, as a positive result may be obtained even in conditions of gross contamination. For more details regarding the use of PCR in detecting insect symbionts, Chapter 46. In addition to the primer sets GP01F and GP01R (Chapter 46), a second set of primers, RLO1F and RLO1R, that are specific for a symbiont genomic repetitive element (Welburn and Gibson, 1989), are very useful for detecting the presence of tsetse secondary endosymbionts:

RLO1F	5′-GAATAATCCCAAGGCTACGC-3′
RLO1R	5′-CCGATTGACTAGGCCTCTAG-3′

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CHAPTER FORTY FIVE

Genetic manipulation of insect symbionts

C. Ben Beard and Serap Aksoy

45.1 INTRODUCTION

45.1.1 Bacterial symbionts as tools for modifying arthropod vector competence

Many haematophagous insects harbour bacterial symbionts, involved either in nutritional mutualism or as reproductive system parasites. Recent studies have demonstrated the feasibility of genetically transforming these agents in an effort to express foreign genes in their host that could potentially alter the host's ability to transmit a disease pathogen (Beard *et al.*, 1993b; Richards, 1993). As the insect–symbiont relationships of other vectors, both of human and plant pathogenic agents, are further explored, it is likely that this approach will find a broad application.

45.1.2 Necessary requirements for a symbiont-mediated approach to vector modification

If these microorganisms are to be utilized for expressing a foreign gene in their host, there are three necessary requirements: (i) a method for collecting large numbers of viable bacteria from the insect; (ii) a method for genetically transforming the bacteria, including a suitable DNA vector; and (iii) a method for replacing the normal symbiont flora with a population of transformed symbionts that are expressing the gene of interest.

Large numbers of viable microorganisms can be obtained either by isolation and purification procedures or by culturing the microbe. Several protocols have been developed for purifying bacterial symbionts directly from insects. Most of them rely on the use of density gradients composed of materials such as sucrose, Ficoll, Percoll, or Renograffin. In a few cases, symbionts can actually be isolated and grown in artificial culture medium (Welburn *et al.*, 1987). A discussion of culture methods applicable to insect endosymbionts is found in Chapter 44.

For transforming bacterial symbionts, two approaches have been successful, both using plasmid transformation systems. The first approach involves the use of broad

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host range plasmids. If information is available regarding the general taxonomic position of the symbiont, it is sometimes possible to use a plasmid that contains a broad host range replication origin that would be compatible in the bacteria. This approach has worked with the secondary bacterial endosymbionts isolated from the tsetse, *Glossina morsitans* (Beard *et al.*, 1993a).

The second approach is based on the observation that many bacteria naturally harbour endogenous plasmids. In such a case, if an ample number of symbionts can be obtained, either directly from their hosts or from *in vitro* cultures, in order to isolate and visualize plasmid DNA on agarose gels, then a shuttle plasmid can be constructed (Figure 45.1). This approach has been successful in experiments with the actinomycete symbiont *Rhodococcus rhodnii* from the Chagas' disease vector *Rhodnius prolixus* (Beard *et al.*, 1992).

The final requirement for establishing a symbiont-based approach to vector modification is the development of a method for replacing the normal bacterial symbiont flora of an insect with a population of modified symbionts. The biological nature of the symbiotic relationship often dictates how replacement might be best approached. In the case of *R. rhodnii*, the symbionts are transmitted from adult to progeny, basically through a faecal-oral route (Dasch *et al.*, 1984). Consequently, newly laid eggs can be surface sterilized and placed into clean cages that do not contain any older

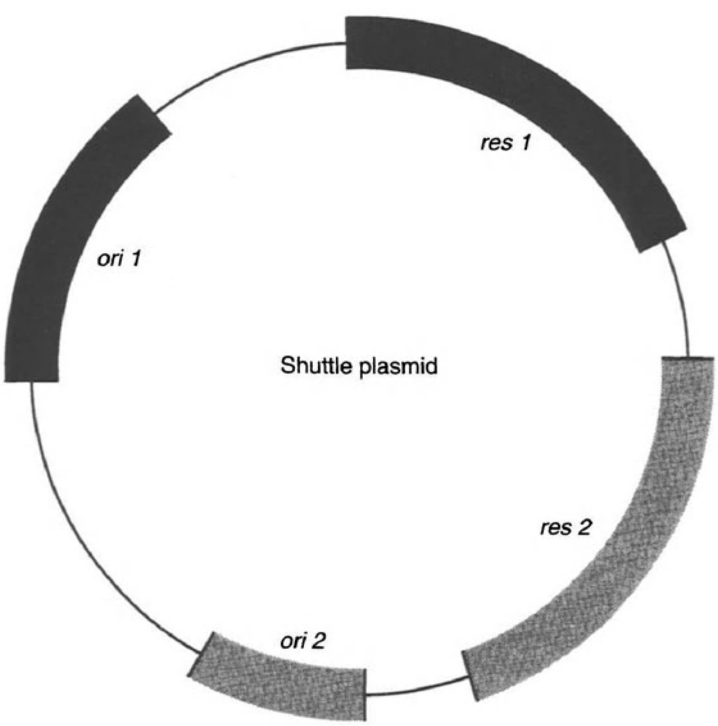


Figure 45.1 A shuttle plasmid is an extrachromosomal DNA element that contains two unique replication origins that allow replication to occur in two very different hosts, usually one of the hosts being *E. coli* and the other being another organism (bacteria, yeast, etc.) where the *E. coli* origin is not recognized. In addition to the independent replication origins (*ori 1* and *ori 2*), the shuttle plasmid must also carry antibiotic resistance genes (*res 1* and *res 2*) that allow for selection in the two different systems. The advantage of a shuttle plasmid over simply using a native plasmid from the heterologous system is that the standard *E. coli* replicons (i.e. ColE1, pMB1) yield very high copy numbers (i.e. up to 3000 copies/cells under certain conditions) in transformed cells; whereas, copy numbers for *Streptomyces* and *Rhodococcus* spp. plasmids can sometimes be as few as 5–10 copies per cell (Hopwood *et al.*, 1985). Additionally, the use of an *E. coli* host allows access to a wealth of kits, reagents, and protocols developed for use in that system which may not be readily applicable to the other systems.

bugs or their faecal material. The nymphs will hatch and remain aposymbiotic. Genetically modified symbionts can be introduced either by contamination of cage materials or by direct feeding through a membrane.

In other insects, it may be necessary to remove nontransformed symbionts by one of several published methods, including antibiotic treatment, high temperatures, or the use of antibodies that have been made against the symbiont (Dasch *et al.*, 1984). Modified symbionts may then be introduced and established with the help of antibiotic selection. Similarly, antibiotic treatment may be used in a one step process for exchanging normal symbionts for modified, antibiotic-resistant symbionts.

In some cases, bacterial symbionts of the genus *Wolbachia* have been introduced by embryo microinjection directly into noninfected insects resulting in the establishment of stable *Wolbachia* infections in a new insect host (Boyle *et al.*, 1993). The transinfected lines demonstrate the cytoplasmic incompatibility (CI) crossing phenotype of their original host. These transinfection experiments have been successful in *Drosophila* using *Wolbachia* derived either from other *Drosophila* lines or from mosquitoes (Braig *et al.*, 1994), and demonstrate the potential of this technology for use with mosquito-borne diseases such as arboviruses and malaria.

The protocols included in this chapter have been used successfully for transforming two quite different groups of bacterial symbionts, the Gram-negative secondary endosymbionts of tsetse, which fall into the γ-3 group of the Proteobacteria and the Gram-positive actinomycete symbiont *R. rhodnii* of the kissing bug *R. prolixus* (Beard *et al.*, 1992, 1993a). The two groups of symbionts are at opposite ends of the spectrum in terms of bacterial phylogeny and host relationship; consequently, together they may suggest the types of approaches that may be tried in other symbiont-host systems.

45.2 MATERIALS

45.2.1 Reagents used for *Rhodococcus* electrotransformation

1. Brain heart infusion (BHI) broth/agar: commercially prepared medium obtained from DIFCO (Detroit, MI), dissolved in ddH_2O according to factory instructions. For agar plates: 1.5% (w/v) bacto agar (DIFCO) added, autoclaved 20 min at 15 psi. Antibiotics for selection can be added sterilely to the agar once it has cooled to around 50°C, just prior to pouring the plates.
2. 10% Glycerol, 0.05% Tween 80: (w/v) concentrations of both reagents added to ddH_2O and autoclaved for 20 min at 15 psi. A second solution of 10% glycerol is prepared in the same manner without the addition of Tween 80 for the final wash and for suspension of the electrocompetent cells.
3. TE: 10 mM Tris, 1 mM EDTA, buffered with HCl to pH 8.0 in ddH_2O and filtered through a 0.2 μm sterilizing filter.

45.2.2 Reagents used for tsetse endosymbiont transformation

1. F buffer: 50 mM calcium chloride, 100 mM potassium chloride, 10 mM potassium acetate, and 10% (w/v) glycerol. This buffer may be autoclaved or filtered through a 0.45 μm sterilizing filter. The final pH should be 6.2.
2. MM medium: commercially prepared Mitsuhashi-Maramorosch medium (Sigma, St. Louis, MO) supplemented with 20% heat-inactivated fetal bovine serum.

3. Antibiotic solution: 20 mg/mL ampicillin stock solution (1000×) in ddH_2O, sterilized using a 0.2 μm sterile syringe filter.

45.3 METHODS

45.3.1 Transformation of *Rhodococcus* symbionts of triatomines

The original protocol that described transformation of *R. rhodnii* (Beard *et al.*, 1992) required first that protoplasts be made from the cells to be transformed. Transformation was facilitated by the use of polyethylene glycol; after which, the fragile cells had to be maintained on a complex medium that would allow cell wall regeneration before selection could be applied. The procedure was both tedious and time-consuming.

The method described here was developed on the basis of a similar approach developed for *Mycobacterium smegmatis* (Jacobs *et al.*, 1991). It is easier, faster, and much more efficient, yielding 3–4 logs more transformants per μg of DNA than the older method which required the use of protoplasts. This particular protocol involves electrotransformation using a Bio-Rad *E. coli* Pulser apparatus (Richmond, CA); however, similar results should be obtainable when the same conditions are met using other electroporators.

(a) Preparation of electrocompetent R. rhodnii *cells*

1. Inoculate 500 mL of BHI medium with a single colony of *R. rhodnii*.
2. Grow the culture at RT with constant shaking until the flask reaches an A_{600} of 0.5–1.0 (3–4 days).
3. Incubate the cells on ice for 1.5 h.
4. Transfer the culture to two 250 mL centrifuge bottles and spin the cells in a Sorval GSA rotor at 5000 **g** for 5 min at 4°C.
5. Decant the supernatant and resuspend the pellet in 250 mL of ice cold 10% glycerol and 0.05% Tween 80.
6. Spin down as before at 5000 **g**.
7. Decant the supernatant and resuspend the pellet in 10 mL of ice cold 10% glycerol and 0.05% Tween 80.
8. Decant the supernatant and resuspend the pellet in 10 mL of ice cold 10% glycerol (no Tween).
9. Spin down as before.
10. Decant the supernatant and resuspend the pellet in 1 mL of ice cold 10% glycerol.
11. Aliquot the cells by dispensing 100–200 μL of electrocompetent cells into chilled microfuge tubes (100 μL if planning to use immediately and 200 μL if planning to freeze).
12. Keep the cells on ice until ready to use; otherwise, quick freeze them in a dry ice/ethanol bath and store at –80°C.

(b) Electrotransformation of competent cells

1. To the microfuge tube that contains 100–200 μL of electrocompetent cells, add 10 ng–5 μg of transformation-quality DNA in TE or ddH_2O to the tube. Mix well

by tapping the tube or gently pipetting and incubate on ice for 1 min. If the DNA that is being used was prepared from *E. coli*, it may be necessary to use concentrations in the higher range due to restriction barriers in *Rhodococcus* that may result in degradation of heterologous DNA (Haynes and Britz, 1990).

2. Transfer 80 μL of electrocompetent cells with DNA to a chilled 0.1 cm gap cuvette (Bio-Rad, Richmond, CA). Tap the cuvette to dislodge any trapped air.
3. Electrotransform the cells using a field strength of 18.5 kV/cm with a 5 ms time constant (the automatic presetting for the BioRad *E. coli* Pulser apparatus when using 0.1 cm gap cuvettes).
4. Add 1 mL of BHI directly to the cuvette, mix, aspirate, and transfer the cells to a 15 mL polypropylene tube.
5. Incubate the cells at RT for 2 h.
6. Spread aliquots of the transformed cells directly onto BHI agar plates that contain the appropriate selective antibiotic (usually thiostrepton or kanamycin).
7. Incubate at 28°C for 3–5 days, observing for growth.

45.3.2 Transformation of Tsetse *S* (secondary)-endosymbionts

1. Inoculate a culture of symbionts in 50 mL of MM medium in a 150 cm^2 tissue culture flask and grow to an A_{600} of approximately 0.3.
2. Centrifuge the cells at 5000 g for 5 min.
3. Decant the supernatant, and resuspend the pellet in 10 mL of ice cold F buffer.
4. Chill the cells on ice for 45 min.
5. Centrifuge the cells again as before and resuspend the pellet in 1 mL of ice cold F buffer.
6. Aliquot 100 μL of competent cells into chilled microfuge tubes on ice.
7. Add 1–10 μg of DNA to an aliquot of cells and incubate on ice for 30 min.
8. Heat shock the cell at 37°C for 45 s, and return the tubes to ice for 2 min.
9. Add 0.9 mL of MM culture medium, and incubate at 28°C overnight.
10. The next day, add ampicillin stock (for plasmids carrying the Ampicillin resistance gene) to a final concentration of 20 μg/mL.

NOTES

1. It is important to use molecular biology grade reagents, including ddH_2O for the preparation of all necessary buffers and solutions required in these procedures.
2. As with other Gram-positive organisms (Haynes and Britz, 1989), the transformation efficiency of *R. rhodnii* can be increased significantly by supplementing the BHI broth with 2% glycine and 0.1% Tween 80 for the preparation of electrocompetent cells.
3. Transformation-quality DNA may be obtained using any of a number of different kits, such as the Wizard Maxiprep DNA kit by Promega (Madison, WI). The DNA can normally be suspended in TE pH 8.0. If arcing occurs during electorporation, the DNA may need to be suspended in ddH_2O.
4. It is very important in both of the protocols described above to include the proper control experiments. These controls should include cells that are mock transformed and then subjected both to antibiotic selection and nonselection;

the former to show that selection kills nontransformed cells, and the latter to show that the cells themselves are viable. A third control should include maintaining one group of transformed cells in the absence of antibiotic selection in order to show that the transformation procedure did not kill the cells.

5. The use of specific trade names does not constitute an endorsement by the US Public Health Service or the Department of Health and Human Services.

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CHAPTER FORTY SIX

PCR-based detection and identification of insect symbionts

Scott L. O'Neill

46.1 INTRODUCTION

Bacterial symbionts of insects are very common and, in many instances, can have profound effects on the physiology of their host insect. Many of them live intracellularly in modified host cells (mycetocytes) and all are transmitted from one insect generation to the next with great fidelity. In some cases these agents are required for the survival of their host, presumably by supplying the insect with required nutrients. Examples of these mutualistic associations include the extracellular symbionts of the triatomine bug, *Rhodnius prolixus* (Baines, 1956), and the intracellular mycetocyte associated bacteria of tsetse flies (Southwood *et al.*, 1975; Aksoy *et al.*, 1995). These symbionts are found in 100% of host insects and their removal by antibiotics can lead to slower growth, sterility and ultimately death. In addition to this traditional class of insect mutualists, a number of examples of bacterial symbionts which exert a more parasitic effect on their insect host have also been described. Among the best studied is the rickettsia-like microorganism *Wolbachia pipientis* (O'Neill *et al.*, 1992). This agent, while providing no direct benefit to its insect host, commonly maintains itself in host populations at extremely high frequencies by drastically lowering the reproductive success of female insects that do not carry the microorganism (O'Neill, 1995). When uninfected females mate with infected males, nearly all of their eggs die due to a process known as cytoplasmic incompatibility. In contrast, infected females are fully compatible with both infected and uninfected males. This indirectly benefits infected females which pass the symbiont to the next generation in their eggs.

The intimate association between insect and symbiont results in an interesting question of autonomy. In many cases the insect cannot survive without its symbionts, and similarly, free-living forms of most symbionts are unknown. In these cases, the insect can be considered to have an extended genome comprised of eukaryotic chromosomes, mitochondrial DNA as well as the genome(s) of symbionts. Historically, most attention has been paid to studying the chromosomal genetics of insects, with very little attention being given to their symbionts, despite the fact that mutualistic associations can be of fundamental importance to the natural physiology of the insect, while some parasitic associations can lead to gross distortions of 'normal' physiology. The manipulation of both types of association may have many applied possibilities (Beard *et al.*, 1993).

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One reason for a lack of attention to these systems has been the fastidious nature of the bacteria. Very few symbionts have been cultured outside of their host in cell free systems, thereby preventing even a traditional bacterial taxonomic placement. The advent of molecular techniques, in particular PCR, has provided a new opportunity for studying many of these interesting systems. Recent studies are showing that many of these associations are much more common than previously thought. For example, five years ago only a handful of insect species were known to be infected with *Wolbachia*. With the results of PCR analysis, we now predict that hundreds of thousands of insect species are naturally infected with this agent, including a surprising number of insect disease vectors.

This chapter provides protocols for the amplification of symbiont genes from complex mixtures of total insect DNA. This approach can be used as a way of diagnosing the presence of known symbiont infections. It can also be used as a method for taxonomic placement of unknown bacterial symbionts by utilizing the sequence of 16S rRNA gene fragments amplified by PCR. Similar techniques can be used to detect human pathogens within insect disease vectors. A comprehensive review on this topic has been published recently (Higgins and Azad, 1995).

46.2 MATERIALS

46.2.1 Sample preparation

1. STE: 0.1 M NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0).
2. Proteinase K: 10 mg/mL in water (stored at –20°C).
3. Plastic reusable homogenizer (Kontes disposable pellet mixer which we clean and reuse).

46.2.2 PCR primers

Three categories of primers are listed below. The first includes general primers which will amplify from nearly any bacterial species, but not from insect DNA. These are useful when dealing with an unidentified symbiont, but care needs to be taken with possible sources of contamination, e.g. surface or midgut contamination. The second category includes a set of positive control primers which will amplify from any insect. These can be used to control for the quality of the DNA preparation. The third category includes some examples of primer sets that have been developed specifically to amplify a given symbiont, and can be used diagnostically to determine infection. Two primers sets are given for *Wolbachia*; the 99F/994R combination is the most specific, while the *ftsZ* set is more sensitive but is not as specific.

1. Nonspecific bacterial primers
   General eubacterial 16S rRNA (Set 1)
   Expected product size ~ 1100 bp (O'Neill *et al.*, 1992):
   61F: 5′-GCTTAACACATGCAAG-3′
   1227R: 5′-CCATTGTAGCACGTGT-3′
   General eubacterial 16S rRNA (Set 2)
   Expected product size ~ 1450 bp (Weisburg *et al.*, 1991):

fD1: 5′-AGAGTTTGATCCTGGCTCAG-3′
rP2: 5′-ACGGCTACCTTGTTACGACTT-3′

2. Control insect primers
   Insect mitochondrial 12S rRNA
   Expected product size ~ 400 bp (Simon *et al.*, 1991):
   12SAI: 5′-AAACTAGGATTAGATACCCTATTAT-3′
   12SBI: 5′-AAGAGCGACGGGCGATGTGT-3′
3. Diagnostic symbiont primer examples
   Wolbachia pipientis diagnostic 16S
   Expected product size ~ 900 bp (O'Neill *et al.*, 1992):
   99F: 5′-TTGTAGCCTGCTATGGTATAACT-3′
   994R: 5′-GAATAGGTATGATTTTCATGT-3′
   Wolbachia pipientis sensitive *ftsZ*
   Expected product size ~ 700 bp (Holden *et al.*, 1993):
   494F: 5′-CCGTATGCCGATTGCAGAGCTTG-3′
   1262R: 5′-TCGCCATGAGTATTCACTTGGCT-3′
   Tsetse midgut symbionts (not-mycetome associated)
   Expected product size ~ 1200 bp (O'Neill *et al.*, 1993):
   GPO1-F: 5′-TGAGAGGTTCGTCAATGA-3′
   GPO1-R: 5′-ACGCTGCGTGACCATTC-3′
   Aphid mycetome symbionts
   Expected product size ~ 800 bp (Rouhbakhsh *et al.*, 1994):
   B1: 5′-GGCGAAAGCCTGATGCAGC-3′
   B2: 5′-GTTTGCTT(AG)TCTTT(AG)CAG(AG)(TG)-3′

46.2.3 PCR reaction

1. *Taq* polymerase.
2. Reaction buffer (supplied with enzyme containing $MgCl_2$ at a final reaction concentration of 2.5 mM).
3. dNTPs 10 mM stock (prepare as a 10 mM solution of each individual base, dATP, dCTP, dGTP and dTTP in ddH_2O as a single solution).
4. Primers (each at a stock concentration of 20 μM).
5. Mineral oil.

46.2.4 Cloning PCR fragments

1. Bluescript-KS® phagemid (Stratagene).
2. *Eco*RV.
3. 1 mM ddTTP.
4. Terminal transferase.
5. T_4 DNA ligase.
6. *E. coli* competent cells (e.g. DH5α).
7. Phenol (Note 2).
8. Ethanol (Note 2).
9. Terminal transferase buffer (10×), commercially available or made up as follows: 1 M potassium cacodylate (pH 7.2), 10 mM $CoCl_2$, 10 mM DTT.

46.3 METHODS

46.3.1 Sample preparation

The following method is a simple and quick procedure for purifying DNA for PCR. For some insect species the DNA produced by this method is not of sufficient quality for PCR. Alternative methods are covered in the Notes section of this chapter.

1. Homogenize whole insect or dissected insect tissue in 100 μL of STE containing 2 μL of proteinase K. Usually 1 mosquito is appropriate for this volume. If using dissected tissue or smaller insects use 1 mosquito equivalent of tissue.
2. Incubate at 55°C for 30 min to allow proteinase K digestion.
3. Incubate at 95°C for 15 min.
4. Spin sample in microcentrifuge at 12 000 **g** for 2 min.
5. Transfer supernatant to new tube and store at 4°C, discard pellet.

46.3.2 PCR reaction

We commonly set up 20 μL PCR reactions and run 10 μL of the reaction on an agarose gel to confirm amplification.

1. For a 20 μL PCR reaction combine 13.5 μL of ddH_2O, 2 μL of 10× reaction buffer, 2 μL of 25 mM $MgCl_2$ stock, 0.5 μL of 10 mM dNTP mix, 0.5 μL of each primer (20 μM), 1 U *Taq* polymerase. Mix well.
2. Add 1 μL of DNA sample, mix.
3. Overlay with 1–2 drops of mineral oil.
4. For all of the primer sets listed here the following reaction conditions apply. An initial denaturation at 95°C for 3 min followed by 35 cycles of 95°C 1 min: 55°C 1 min: 72°C 1 min.
5. Run 10 μL of PCR reaction on a 1% agarose gel in 0.5× TBE.

46.3.3 Cloning PCR products

In order to determine the phylogenetic position of a given agent it is necessary to clone the 16S rRNA PCR product and sequence a number of clones. We routinely use TA cloning of PCR products and make the vector ourselves (Holton and Graham, 1991); however commercial TA cloning vectors can be purchased (e.g. Stratagene).

1. Digest 5 μg of pBluescript KS^- with *Eco*RV at 37°C for 3 h.
2. Heat kill enzyme at 65°C for 10 min and ethanol precipitate DNA, and dry pellet.
3. Resuspend precipitated and dried DNA into a 40 μL reaction containing 100 mM potassium cacodylate (pH 7.2), 1 mM $CoCl_2$, 1 mM DTT, 10 mM ddTTP and 100 U of terminal transferase; incubate for 1 h at 37°C.
4. Phenol extract and precipitate T-tailed phagemid DNA and resuspend in 20 μL of H_2O.
5. Set up a 10 μL ligation reaction containing at least equimolar amounts of phagemid and PCR product (usually 1μL of a 20 μL PCR reaction is added directly to the ligation reaction) and incubate at 16°C for at least 5 h.

6. Use 2 μL of the ligase reaction to transform competent *E. coli* cells (e.g. DH5α), plate onto LB agar plates supplemented with ampicillin, IPTG and X-gal (Chapter 25). Select white colonies, miniprep and size select plasmids with the correct sized insert.
7. Sequence inserts using standard sequencing protocols (Chapter 24).

46.3.4 Phylogenetic placement of symbionts

The easiest method to quickly determine a phylogenetic placement for an unknown bacterial symbiont is to submit the sequence to the RDP database (Maidak *et al.*, 1994). This database is accessible through the internet by directing a WWW browser, such as Netscape, to the following URL (http://rdp.life.uiuc.edu/). The RDP provides an easy to use interface for submitting sequences, retrieving phylogenetic trees and other useful information.

NOTES

1. Problems with PCR amplification: In some cases the quick protocol for DNA preparation does not produce DNA of high enough quality for good PCR amplification. A common indicator of this is the inability to amplify a fragment with the 12SAI and 12SBI control primer set. This is almost never a problem with dissected tissue, but can be when whole insects of certain species are used, e.g. *Aedes albopictus*. It appears that inhibitors may be present in the cuticle of these species. One easy solution that often works is to use whole abdomens instead of whole insects. These are prepared by using a clean razor blade to sever them from the thorax of the desired insect. Alternatively, carefully dissecting tissues of interest under clean conditions and homogenizing them will fix the problem. In addition a more thorough DNA preparation can be used, although these are often more time-consuming to perform (Chapter 14 gives more information).
2. It is essential that molecular biological-grade reagents are used in all of these procedures.

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